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Los Angeles

Characterizing the stabilizing effect of the putative kinase Coq8 and the function of the Coq9 polypeptide in yeast coenzyme Q biosynthesis

A dissertation submitted in partial satisfaction of the requirements for

the degree Doctor of Philosophy

in Biochemistry and Molecular Biology

by

Cuiwen He

2015

ABSTRACT OF THE DISSERTATION

Characterizing the stabilizing effect of the putative kinase Coq8 and the function of the Coq9 polypeptide in yeast coenzyme Q biosynthesis

by

Cuiwen He

Doctor of Philosophy in Biochemistry & Molecular Biology

University of California, Los Angeles, 2015

Professor Catherine F. Clarke, Chair

Coenzyme Q (Q) is an essential lipid in cellular energy metabolism, but its biosynthesis is not fully understood. Q functions as an electron carrier in the mitochondrial respiratory chain and as a lipid-soluble antioxidant. Q biosynthesis in yeast *Saccharomyces cerevisiae* requires a multi-subunit Coq polypeptide complex composed of the Coq3−Coq9 polypeptides, but the function of several Coq polypeptides is unknown, including Coq9. Deletion of any of the *COQ3−COQ9* genes leads to the decreased steady state of other Coq polypeptides. The over-expression of the putative kinase, Coq8, in some of the yeast *coq* null mutants, restored steady state levels of Coq polypeptides to near wild-type levels and led to the production of late-stage Q intermediates. In this dissertation, the following chapters summarize four projects on Q biosynthesis: Chapter 2 investigates whether Coenzyme Q_6 supplementation or over-expression of Coq8 stabilizes high molecular mass Coq polypeptide complexes. Based on our findings, we proposed a new model for the complex, which we called the CoQ-synthome. In Chapter 3, the characterization of Coq9 function is described. We conclude that Coq9 is required for the function of Coq6 and Coq7 and

for the removal of the nitrogen substituent from Q-intermediates derived from *para*-aminobenzoic acid. The functional role of human Coq9 in Q_{10} biosynthesis is not understood. In Chapter 4 we found that human *COQ9* rescues the growth of a temperature-sensitive yeast *coq9* mutant, TS19, on non-fermentable carbon source and increases the content of Q_6, possibly by increasing the Q biosynthesis from 4-hydroxybenzoic acid (4HB). Chapter 5 demonstrates that *para*-coumarate is a ring precursor for Q biosynthesis in *E. coli*, *S. cerevisiae*, and human cells. This work aids our understanding of Q biosynthesis and suggests new approaches that may enhance Q biosynthesis and function in human disease.

The dissertation of Cuiwen He is approved.

Jorge Torres

Alexander M. van der Bliek

Catherine F. Clarke, Committee Chair

University of California, Los Angeles

2015

To my dearest grandmother,

whom I love and miss always and forever.

奶奶，

我永远爱您和怀念您。

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Publications and Presentations

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Chapter 1

Introduction to Coenzyme Q and its Biosynthesis

What is Coenzyme Q?

Coenzyme Q (ubiquinone, CoQ or Q) is a lipid that present in all eukaryotes and α- β- and γ-proteobacteria (1). Q was first discovered in mitochondria, but it also presents in endoplasmic reticulum, Golgi apparatus, lysosomes, peroxisomes, nuclear envelope and plasma membrane (2-4). It is composed of a redox active benzoquinone ring and a polyisoprenyl tail, which anchors Q to cellular membranes (5). Q is located in the membrane midplane and its head group oscillates across the membrane (6). The quinone head group provides redox function. Ubisemiquinone radical ($QH^{\bullet}$) is produced when Q accepts one electron and one proton, and ubiquinol (QH_2) is produced when the second electron and proton are accepted. The benzoquinone ring is conserved, while the length of the polyisoprenoid side chain varies in different organisms; for example, *Saccharomyces cerevisiae* has six isoprene units (Q_6), *Escherichia coli* has eight units (Q_8), and humans have ten subunits (Q_{10}) (5). Q can be found in the forms of Ubiquinone-1 to Ubiquinone-11 (7-9). The quinone part of Q is thought to be more important than the side chain for Q function. It has been shown that Q_9 can replace the function of Q_8 in *E. coli* and there are no functional differences of Q_5–Q_{10} in complementation in yeast (10-12). However, different organisms do prefer their original Q species and tail length may affect Q function (12-14). It was speculated that there is correlation between the side chain length of Q and the hydrophobicity of the organism's membrane (12).

Q plays an essential role as an electron carrier in the mitochondrial respiratory chain by accepting electrons from Complex I and Complex II and donating electrons to Complex III. Complex I (rotenone-sensitive NADH-ubiquinone oxidoreductase) oxidizes NADH and reduces Q with the coupling of proton translocation from the matrix to the mitochondrial intermembrane space (15). Different from bacteria and most eukaryotes, *S. cerevisiae* and many other yeasts use

rotenone-insensitive NADH-ubiquinone oxidoreductase instead of complex I and there is no proton translocation coupling (16). Complex II (succinate:quinone oxidoreductase) oxidizes succinate to fumarate and transfers electrons to Q, but it does not translocate protons (15). Next, QH_2 transfers electrons to cytochrome *c* at complex III (the cytochrome bc_1 complex) through the Q cycle. Complex III contains a di-heme cytochrome *b*, cytochrome c_1, and an iron-sulfur protein known as the Rieske protein. At the positive side of the membrane (center P), ubiquinol (QH_2) is oxidized. One electron is transferred from QH_2 to the 2Fe:2S cluster of the Rieske protein, resulting the formation of ubisemiquinone radical anion ($Q^{-\bullet}{}_P$), which immediately reduces the low potential heme of cytochrome *b* (heme b_L). In this process, two protons are released to the outer P side of the membrane and two electrons transferred. One of these electrons is transferred to cytochrome *c* from the Rieske protein and the other one is transferred to the high potential heme of cytochrome *b* (heme b_H), where it reduces Q to form ($Q^{-\bullet}{}_n$) at the negative side of the membrane, N side. At this point, the first half of the Q cycle is completed. The process above is repeated when a second QH_2 is oxidized; however, heme b_H reduces the $Q^{-\bullet}{}_n$ to QH_2 and two protons are consumed from the inner side of the membrane at the last step, and this is the second half of the Q cycle. In summary, each Q cycle results in the oxidation of one QH_2, reduction of two molecules of cytochrome *c*, consumption of two protons on the N side of the membrane and release of four protons on the P side of the membrane (17).

Another important function of Q/QH_2 is its role as the only lipid soluble antioxidant that is synthesized endogenously (3). QH_2 interferes both the initiation and propagation of lipid oxidation and protects proteins and DNA from the damage caused by oxidative stress (3). Q is synthesized abundantly in every cell and is reduced continuously, so it can respond to oxidative stress immediately and effectively (3,18). Furthermore, Q regenerates vitamin E from α-

tocopheroxyl radical (19).

Q also has many other functions (18). By scavenging free radicals Q inhibits apoptosis ; Q also prevents apoptosis independent of free radicals by inhibiting mitochondrial permeability transition pore opening (20). Q is a cofactor for fatty-acid-dependent proton transport by uncoupling proteins, UCP1, UCP2, and UCP3 (21,22). Q has inducible effects on NFκ B-regulated genes, which are important in inflammatory response (23). Q regulates the physicochemical properties of membranes, it modulates the amount of h2-integrins on the surface of blood monocytes, it improves the endothelial dysfunction, it oxidizes sulfide in yeast, and it introduces disulfide bonds in bacteria (19).

The biosynthesis of Coenzyme Q

The Q biosynthesis pathway is highly conserved in different organisms, and the yeast *S. cerevisiae* has been an important model to study Q biosynthesis. In *S. cerevisiae*, 4-hydroxybenzoate (4-HB) and *para*-aminobenzoic acid (pABA) are two known aromatic precursors of the head group of Q (24,25). Different organisms produce 4-HB from different sources: animals produce 4-HB from tyrosine, *E. coli* utilizes chorismate, and yeast uses either tyrosine or shikimate (25). pABA is a precursor of folates and it is derived from chorismate in *E. coli* and yeast (26). The precursor for the polyisoprenoid side chain of Q is farnesyl-PP, a molecule produced from acetyl-CoA in the mevalonate pathway (27).

Eleven gene products are required in Q biosynthesis in *S. cerevisiae*: Coq1−Coq9, Arh1, and Yah1, and yeast with deletion of any of the *COQ1−COQ9* genes fail to synthesize Q and are not able to respire (24,28). The functions of Coq1, Coq2, Coq5, Coq6, and Coq7 have been identified and their involvement in the Q biosynthesis pathway is described in Figure 1.

Coq1 is a polyprenyl diphosphate synthase, which synthesize the polyisoprenoid side chain of Q and determines the length of the polyprenyl tail in different organisms (10). Coq1 is peripherally associated with the inner membrane facing the matrix side in yeast mitochondria (29). Coq1 has homologs in various organisms. In *E. coli*, the octaprenyl-diphosphate synthase, IspB, froms a homodimer to catalyze the synthesis of the side chain (30). In *Arabidopsis thaliana,* solanesyl diphosphate synthase, SPS1, is a homomeric enzyme that synthesizes the polyisoprenoid side chains in the ER, which are transferred to the mitochondria for Q biosynthesis (31). Coq1 homologs function as heterotetramer in some organisms: PDSS1 and PDSS2 are the subunits in the polyprenyl diphosphate synthases of fission yeast, mouse, and human (28).

Coq2, the 4-HB polyprenyltransferase, is an integral membrane protein in the inner mitochondrial membrane and it transfers the polyisoprenoid side chain to the ring precursors, generating the intermediates 3-hexaprenyl-4-aminobenzoic acid (HAB) (with pABA as precursor) or 3-hexaprenyl-4-hydroxybenzoic acid (HHB) (with 4-HB as precursor). Coq2 does not have specificity for the lengths of the polyprenyl tails (32). *S. cerevisiae* Coq2 has homologs in *E. coli* (encoded by *ubiA*), *S. pombe* (encoded by *ppt1*), *A. thaliana* (encoded by AtPPT1), and *Homo sapiens* (encoded by *COQ2*) (32-36).

In *S. cerevisiae*, Coq3–Coq9 form a Q biosynthetic complex that is associated peripherally with the inner membrane on the matrix side and catalyzes the rest of the steps in Q biosynthesis (37). Coq6 (UbiI in *E. coli*) is a flavin-dependent monooxygenase that catalyzes the C5-hydroxylation reaction (38,39). Ferredoxin Yah1 and ferredoxin reductase Arh1 are required as electron donors for Coq6's function (40). FDX1L and FDXR are the human homologs of Yah1 and Arh1 respectively. They provide electrons to CQO6 but are also involved in other

pathways in human (41). Coq3 (UbiG in *E. coli*) catalyzes the *O*-methylation at both the C5 and C6 positions using *S*-adenosylmethionine as the methyl donor (42,43). Coq5 (UbiE in *E. coli*) catalyzes the C2-methylation reaction on 2-methoxy-6-polyprenyl-1,4-benzoquinone, generating 2-methoxy-5-methyl-6-polyprenyl-1,4-benzoquinone (42,44). Coq7 (*clk-1* in *C. elegans)* functions as a 5-demethoxyubiquinone hydroxylase that belongs to a family of di-iron binding oxidases containing a conserved sequence motif for the iron ligands (45). In *E. coli*, UbiF, a flavin-dependent monooxygenases, catalyzes the hydroxylation at C6 of the DMQ_8 (46). Recently, Coq7 is also found to localize in the nucleus and have a functional role in stress responses and longevity that is conserved in *C. elegans* and human. It was demonstrated that Clk-1or Coq7 modulates reactive oxygen species metabolism and unfolded protein response, possibly by regulating gene expression (47).

The enzymatic functions of Coq4, Coq8, and Coq9 in Q biosynthesis are still not clear. Coq4 contains a conserved zinc ligand motif HDxxH-$(x)_{11}$-E, but the sequence of *COQ4* does not have homology to any proteins that have characterized functions (48). The crystal structure for Alr8543, a Coq4 homolog in the cyanobacteria *Nostoc* sp. *PCC7120* has been solved and shown to be a homodimer. Each monomer contains a binding site for the polyisoprenoid side chain (49). Coq8 (UbiB in *E. coli*; ADCK3 and ADCK4 in human) is a putative kinase that belongs to an ancient atypical kinase family (50). It has six of the twelve motifs present in canonical protein kinases, I, II, III, VIB, VII, and VIII motifs (51), and the phosphorylation of Coq3, Coq5, and Coq7 depends on Coq8 (52). The Q-biosynthetic complex comprised of Coq3−Coq9 appears to be affected by Coq8 directly or indirectly. In *coq3−coq9* null mutants, the steady state levels of Coq4, Coq6, Coq7 and Coq9 are destabilized and only early-stage intermediates, HHB and HAB, are accumulated. The over-expression of *COQ8* restores the levels of the destabilized

proteins and leads to the accumulation of diagnostic/late-stage intermediates in most *coq* null yeast mutants (53). The results suggest that the over-expression of *COQ8* stabilizes the multi-subunit Q biosynthetic complex, which is destabilized upon the deletion of any of the *COQ3−COQ9* genes. In Chapter 2, I tested this hypothesis by expressing muti-copy Coq8 in different yeast *coq* null mutants and the Coq polypeptide complex was analyzed with two-dimensional blue native/SDS PAGE followed by Western blot. Coq9 does not have a homolog in *E. coli*, but it does in human. The crystal structure of human Coq9 is solved and identified as a member of an ancient protein family TFR (TetR family of regulators) with a canonical amino terminal helix-turn-helix (HTH) domain. Human Coq9 forms a dimer and the interface of the dimer binds lipids (54). In yeast *coq9* null mutant with the over-expression of Coq8, late-stage intermediates accumulate, including demethoxy-Q_6 (DMQ_6) (when 4HB is the ring precursor), and imino-demethoxy Q_6 ($IDMQ_6$) (when pABA is the ring precursor) (53). With Coq8 over-expression, DMQ_6 also accumulates in the yeast *coq7* null mutant (55), indicating that Coq9 is required for Coq7 function. With Coq8 over-expressed, both *coq6* null and *coq9* null mutants accumulate 4-HP and 4-AP (53), suggesting that Coq9 is also necessary for the function of Coq6. The accumulation of 4-AP and $IDMQ_6$ in yeast *coq9* mutants provided with pABA as a ring precursor suggests that Coq9 is required for the deamination of Q-intermediates. In Chapter 3, I generated a temperature-sensitive *coq9* point mutant and studied the mutations' effect on the steady state levels of Coq polypeptides and Q_6 intermediates accumulated.

Coq10 and Coq11 are the other two Coq proteins that are involved in yeast Q biosynthesis. Yeast with the deletion of either Coq10 or Coq11 still produce Q_6 and are able to grow on non-fermentable carbon source, but their *do novo* Q biosynthesis is less efficient than the wild type (37,56). The structure of Coq10 homolog CC1736 in *Caulobacter crescentus*

shows a steroidogenic acute regulatory protein-related lipid transfer (START) domain and purified CC1736 binds to Q with different polyiosprenyl tail lengths (56). Coq10 may serve as a chaperone that enables Q to function properly by transporting it to complexes of the respiratory electron transport chain. In human, *COQ10A* and *COQ10B* are the two orthologues for *COQ10*. Coq11 is found to co-purify with the Q biosynthetic complex, suggesting its association with the complex, but its function is still not clear (37).

Coenzyme Q and human diseases

Coenzyme Q is an essential lipid that functions in many aspects of cellular processes and Q_{10} deficiency is associated with various clinical phenotypes. There are two kinds of Q deficiency: primary Q_{10} deficiency, which is caused by mutations in Q_{10} biosynthetic genes, and secondary Q_{10} deficiency, which is caused by nongenetic factors or mutations in genes that are not directly involved with the biosynthesis of Q_{10} (57). In human, there are 15 genes involved in Q_{10} biosynthesis: *PDSS1* and *PDSS2, COQ2, COQ3, COQ4, COQ5, COQ6, COQ7, ADCK3* and *ADCK4, COQ9, COQ10A* and *COQ10B, FDX1L,* and *FDXR* (41). Primary Q_{10} deficiencies are very rare. Mutations in eight of these genes have been reported. Mutations in *PDSS1* caused encephalopathy, peripheral neuropathy, optic atrophy, heart valvulopathy, mild lactic acidosis, and mutations in *PDSS2* caused Leigh syndrome, ataxia, deafness and retinopathy. Mutations in *COQ2* are associated with encephalomyopathy, hypertrophic cardiomyopathy, MELAS-like syndrome, seizures, retinopathy, lactic acidosis, deafness, and adult-onset multisystem atrophy. *COQ4* mutations caused encephalomyopathy and *COQ6* mutations caused deafness, encephalopathy, and seizures. *ADCK3* mutations caused cerebellar ataxia, encephalopathy, seizures, dystonia, and spasticity, and *ADCK4* mutations caused mental retardation. Mutations in

COQ9 caused encephalomyopathy, renal tubulopathy, and cardiac hypertrophy (41). Secondary Q_{10} deficiencies are more common. They are caused by mutant genes unrelated to Q_{10} biosynthesis. Patients with these gene defects do not always develop Q_{10} deficiencies. It is still not clear why some patients are more susceptible. Skeletal muscle and the central nervous system (CNS) affected by Q_{10} deficiencies have been reported. Symptoms of Q_{10} deficiencies in skeletal muscle include weakness, hypotonia, exercise intolerance, and myoglobinuria. Symptoms in CNS defects include ataxia and general CNS impairment (41).

The current treatment for both primary and secondary Q_{10} deficiencies is oral Q_{10} supplementation (57). Primary deficiencies have better response to the treatment, but the bioavailability of Q_{10} is very low (41). Increasing endogenously synthesized Q_{10} would be a more effective treatment. Bezafibrate is a drug used to treat hyper lipidemia, but it was also shown to treat mitochondrial myopathy by inducing mitochondrial biogenesis (58). However, it does not seem to increase Q_{10} biosynthesis (59). Using 4-hydroxybenzoic acid analogues that bypass enzymatic defects in Q_{10} biosynthetic proteins may provide a more promising therapeutic option. Feeding vanillic acid or 3,4-dihydroxybenzoic acid to yeast *coq6* null mutant expressing the mutant huCOQ6-isoa proteins, gene products of a mutant *COQ6* isoform found in patients, restored the yeast's respiratory growth (Fig. 1) (60). It was shown that 2,4-dihydroxybenzoic acid restores Q_6 biosynthesis and the respiratory growth of *coq7* null mutant over-expressing Coq8 (Fig. 1) (53). Recently, a study showed that 2,4-dihydroxybenzoic acid significantly increased the levels of Q_9 in a mouse model carrying a homozygous mutation in *Coq9* gene ($Coq9^{R239X}$), a mutation that is homologue to the human R244X mutation, and elevated the Q_{10} levels in human $COQ9^{R244X}$ skin fibroblasts (61). It is important to understand the functions of the Q_{10} biosynthetic proteins and to elucidate the steps in Q_{10} biosynthesis pathway, so we can use

bypass therapy to treat patients with primary Q_{10} deficiency more effectively. Yeast will be a great model to study human Coq proteins. Human *PDSS1* and *PDSS2, COQ2, COQ3, COQ4, COQ5, COQ6, COQ7, ADCK3* and *ADCK4, COQ10A* and *COQ10B* have been shown to complement the corresponding yeast null mutants (41,62). In Chapter 4, I will describe the rescue of a *coq9* yeast mutant by human *COQ9*.

Although different organisms have similar Q biosynthesis pathways, it is also important to understand their differences so we can expect some of the limitation of using yeast as a model to study human Q biosynthesis. An example is that yeast can use both pABA and 4-HB as aromatic ring precursors for Q biosynthesis, while pABA is an inhibitor of Q biosynthesis in mammalian cells (41). This indicates that yeast harbor an enzyme that can function as a deaminase, which is a function that may not present in mammals. In Chapter 5, I will describe a study on whether different compounds can serve as ring precursors for Q biosynthesis in *E. coli*, *S. cerevisiae*, and mammalian cells.

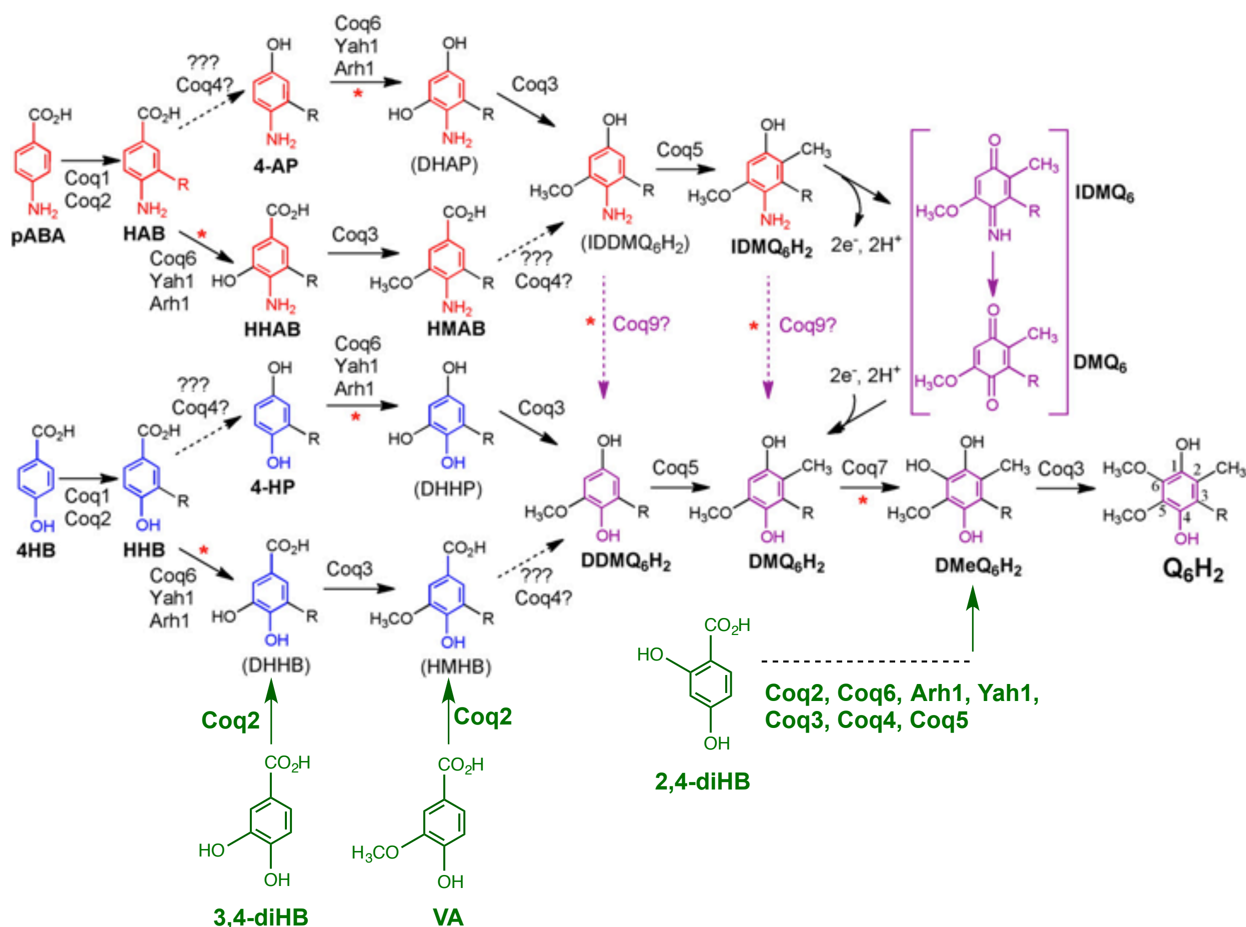

Figure 1. Proposed Q_6 biosynthesis pathway in *S. cerevisiae*. 4HB or pABA are the two known ring precursor for Q biosynthesis in yeast. Intermediates that derive from 4HB are show in blue and intermediates that derive from pABA are shown in red. Purple colored intermediates indicate the convergence of the two pathways. *R* represents the hexaprenyl tail present in Q_6 and all intermediates. Coq1 synthesizes the hexaprenyl-diphosphate tail and Coq2 transfers the tail to 4HB or pABA to form HHB (3-hexaprenyl-4-hydroxybenzoic acid) and HAB (3-hexaprenyl-4-aminobenzoic acid), respectively. Coq6 then catalyzes hydroxylation at C5 with ferredoxin (Yah1) and ferredoxin reductase (Arh1). Coq3 catalyzes the two *O*-methylation steps at C5 and C6 positions, Coq5 performs the *C*-methylation step at C2, and Coq7 puts the hydroxyl group at C6. The functions of Coq4, Coq8, and Coq9 are not clear. *Purple dotted arrows* designate the

proposed C4-deamination/deimination reaction catalyzed by Coq9. *Red asterisks* designate the steps defective in *coq9* null mutant. Intermediates that have been detected are shown in bold and intermediates that have not been detected are shown in parentheses. The bypass of Q biosynthesis steps with 3,4-dihydroxybenzoic acid (3,4-diHB), vanillic acid (VA), or 2,4-dihydroxybenzoic acid (2,4-diHB) is indicated in green. Figure modified from He CH et al., 2014 (63).

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Chapter 2

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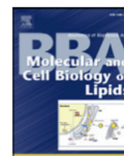

Coenzyme Q supplementation or over-expression of the yeast Coq8 putative kinase stabilizes multi-subunit Coq polypeptide complexes in yeast *coq* null mutants ☆

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ABSTRACT

Coenzyme Q biosynthesis in yeast requires a multi-subunit Coq polypeptide complex. Deletion of any one of the *COQ* genes leads to respiratory deficiency and decreased levels of the Coq4, Coq6, Coq7, and Coq9 polypeptides, suggesting that their association in a high molecular mass complex is required for stability. Over-expression of the putative Coq8 kinase in certain *coq* null mutants restores steady-state levels of the sensitive Coq polypeptides and promotes the synthesis of late-stage Q-intermediates. Here we show that over-expression of Coq8 in yeast *coq* null mutants profoundly affects the association of several of the Coq polypeptides in high molecular mass complexes, as assayed by separation of digitonin extracts of mitochondria by two-dimensional blue-native/SDS PAGE. The Coq4 polypeptide persists at high molecular mass with over-expression of Coq8 in *coq3*, *coq5*, *coq6*, *coq7*, *coq9*, and *coq10* mutants, indicating that Coq4 is a central organizer of the Coq complex. Supplementation with exogenous Q_6 increased the steady-state levels of Coq4, Coq7, and Coq9, and several other mitochondrial polypeptides in select *coq* null mutants, and also promoted the formation of late-stage Q-intermediates. Q supplementation may stabilize this complex by interacting with one or more of the Coq polypeptides. The stabilizing effects of exogenously added Q_6 or over-expression of Coq8 depend on Coq1 and Coq2 production of a polyisoprenyl intermediate. Based on the observed interdependence of the Coq polypeptides, the effect of exogenous Q_6, and the requirement for an endogenously produced polyisoprenyl intermediate, we propose a new model for the Q-biosynthetic complex, termed the CoQ-synthome.

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1. Introduction

Coenzyme Q (ubiquinone, CoQ or Q) is a lipid composed of a fully substituted benzoquinone ring and a polyisoprenyl chain, which contains six isoprene units in *Saccharomyces cerevisiae* (Q_6), eight in *Escherichia coli* (Q_8), and ten in humans (Q_{10}) [1]. Q is an electron carrier in the mitochondrial respiratory chain, and is essential in cellular energy metabolism [2]. The oxidized quinone (Q) accepts electrons from NADH via complex I, or succinate via complex II, and the reduced hydroquinone (QH_2) donates electrons to cytochrome *c* via complex III. Instead of complex I, *S. cerevisiae* rely on the much simpler NADH:Q oxidoreductases that oxidize NADH external to the mitochondria (Nde1 and Nde2), or inside the matrix (Ndi1) [3]. In mammalian mitochondria Q functions to integrate the respiratory chain with many aspects of metabolism by serving as an electron acceptor for glycerol-3-phosphate, dihydroorotate, choline, sarcosine, sulfide, and several amino acid and fatty acylCoA dehydrogenases [4,5]. QH_2 also functions as a crucial lipid-soluble antioxidant [6] and decreased levels of Q are associated with mitochondrial, cardiovascular, kidney and neurodegenerative diseases [7–11]. A better understanding of the enzymatic steps and organization of the polypeptides and cofactors

Abbreviations: 4-AP, 3-hexaprenyl-4-aminophenol; Coq1, the Coq1 polypeptide; *COQ1*, designates the wild-type gene encoding the Coq1 polypeptide; *coq1*, designates a mutated gene; $DDMQ_6$, the oxidized form of demethyl-demethoxy-Q_6H_2; DMQ_6, demethoxy-Q_6; DMQ_6H_2, demethoxy-Q_6H_2; 4-HB, 4-hydroxybenzoic acid; HAB, 3-hexaprenyl-4-aminobenzoic acid; HHAB, 3-hexaprenyl-4-amino-5-hydroxybenzoic acid; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; HMAB, 3-hexaprenyl-4-amino-5-methoxybenzoic acid; 4-HP, 3-hexaprenyl-4-hydroxyphenol; $IDMQ_6$, 4-imino-demethoxy-Q_6; MRM, multiple reaction monitoring; pABA, *para*-aminobenzoic acid; Q, ubiquinone or coenzyme Q; Q_6H_2, ubiquinol or coenzyme Q_6H_2; RP-HPLC-MS/MS, Reverse phase-high performance liquid chromatography–tandem mass spectrometry; START, steroidogenic acute regulatory protein-related lipid transfer

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required for Q biosynthesis will aid efforts to determine how the content of this important lipid can be regulated for optimal metabolism and health.

Q biosynthesis in *S. cerevisiae* requires at least eleven proteins, Coq1–Coq9, Arh1, and Yah1 (Fig. 1) [12–14]. Yeast mutants lacking any of the Coq1–Coq9 polypeptides are respiratory deficient due to the lack of Q. The Coq1 polypeptide synthesizes the hexaprenyl diphosphate tail, and Coq2 attaches the tail to either 4-hydroxybenzoic acid (4HB) or para-aminobenzoic acid (pABA); both are used as aromatic ring precursors in the biosynthesis of Q in yeast [13,15]. The other Coq polypeptides catalyze ring modification steps including *O*-methylation (Coq3), *C*-methylation (Coq5), or hydroxylation (Coq6 and Coq7). Coq6 requires ferredoxin (Yah1) and ferredoxin reductase (Arh1), which presumably serve as electron donors for the ring hydroxylation step [13,16]. Coq4, Coq8, and Coq9 polypeptides are essential for Q biosynthesis but their functional roles are not yet completely understood. In the Q-biosynthetic pathway proceeding from pABA, Coq9 is required for the replacement of the ring amino substituent with a hydroxyl group, although it remains uncertain exactly how this step is carried out [17].

Both genetic and physical evidence indicate that a multi-subunit Coq polypeptide complex is essential for Q biosynthesis [12,18–20]. Deletion of any one of the *COQ* genes in *S. cerevisiae* leads to destabilization of several other Coq polypeptides; the levels of Coq4, Coq6, Coq7, and Coq9 polypeptides are significantly decreased in each of the *coq1–coq9* null mutant yeast strains [20]. Although steady-state levels of the Coq3 polypeptide were also found to be decreased [20], Coq3 levels in mitochondria isolated from the *coq4–coq9* null mutants were shown to be preserved in subsequent studies performed in the presence of phosphatase and protease inhibitors [17,21]. As a result of the interdependence of the Coq polypeptides, *coq3–coq9* null mutant yeast accumulate only the early intermediates 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB), produced by the prenylation of 4HB and pABA, respectively (Fig. 1) [17]. Whereas each of the *coq* null mutants lacks the designated Coq polypeptide [20], several *coq* mutants harboring certain amino acid substitution mutations show a less drastic block in Q biosynthesis as compared to *coq* null mutants. For example, certain *coq7* point mutants retain steady-state levels of the Coq7 polypeptide and accumulate demethoxy-Q_6 (DMQ_6), a late-stage Q-intermediate missing just one methoxy group [22,23]. Some of the Coq polypeptides physically interact — biotinylated Coq3 co-purifies with Coq4 [18], and Coq9 tagged with the hemagglutinin epitope co-purifies with Coq4, Coq5, Coq6, and Coq7 polypeptides [20]. These studies were performed with

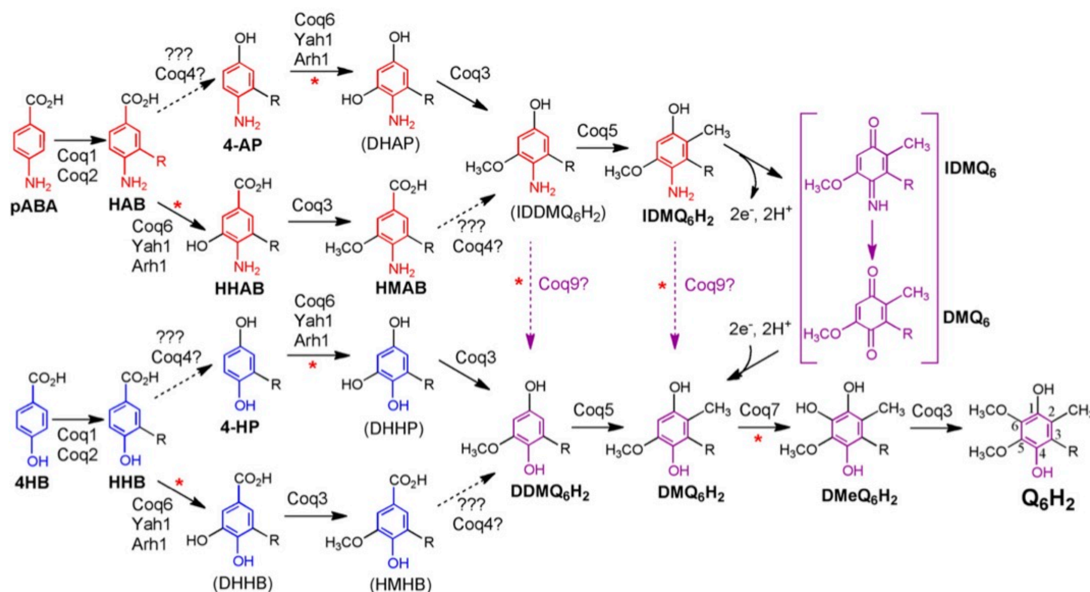

Fig. 1. Q_6 biosynthesis in *S. cerevisiae* proceeds from either 4HB or pABA. The classic Q biosynthetic pathway is shown in *blue* emanating from 4HB (4-hydroxybenzoic acid). *R* represents the hexaprenyl tail present in Q_6 and all intermediates. The numbering of the aromatic carbon atoms used throughout this study is shown on the reduced form of Q_6, Q_6H_2. Coq1 synthesizes the hexaprenyl-diphosphate tail, which is transferred by Coq2 to 4HB to form HHB (3-hexaprenyl-4-hydroxybenzoic acid). Alternatively, the *red* pathway indicates that pABA (*para*-aminobenzoic acid) is prenylated by Coq2 to form HAB (3-hexaprenyl-4-aminobenzoic acid). Both HHB and HAB are early Q_6-intermediates, readily detected in each of the *coq* null strains (Δ*coq3*–Δ*coq9*). Subsequent ring modification steps are thought to occur in the sequences shown, including hydroxylation by Coq6 in concert with ferredoxin (Yah1) and ferredoxin reductase (Arh1). Coq3 performs the two O-methylation steps, Coq5 the C-methylation step, and Coq7 performs the penultimate hydroxylation step. The functional roles of the Coq4, Coq8, and Coq9 polypeptides are elaborated in this study. Coq8p over-expression (*hcCOQ8*) in certain Δ*coq* strains leads to the accumulation of novel intermediates [17], suggesting these branched pathways. For example, in the presence of hcCOQ8, *coq6* or *coq9* mutants accumulate 4-AP (derived from pABA), and 4-HP (derived from 4HB) [17], indicating that in some cases decarboxylation and hydroxylation at position 1 of the ring may precede the Coq6 hydroxylation step. *Purple dotted arrows* designate the replacement of the C4-amine with a C4-hydroxyl and correspond to a proposed C4-deamination/deimination reaction, resulting in a convergence of the 4HB and pABA pathways. A putative mechanism to replace the C4-imino group with the C4-hydroxy group is shown in *purple brackets* for $IDMQ_6$ but could also occur on $IDDMQ_6$ (*not shown*). Several steps defective in the Δ*coq9* strain are designated with *red asterisks*. Intermediates previously detected are shown in bold: 4-AP (3-hexaprenyl-4-aminophenol); $DDMQ_6H_2$, the reduced form of demethyl-demethoxy-Q_6; DMQ_6, demethoxy-Q_6; DMQ_6H_2, demethoxy-Q_6H_2 (the reduced form of DMQ_6); HHAB, 3-hexaprenyl-5-hydroxy-4-aminobenzoic acid; HMAB, 3-hexaprenyl-5-methoxy-4-aminobenzoic acid; 4-HP (3-hexaprenyl-4-hydroxyphenol); $IDMQ_6$, (4-imino-demethoxy-Q_6); $IDMQ_6H_2$, 4-amino-demethoxy-Q_6H_2 (the reduced form of $IDMQ_6$). Intermediates shown in parentheses indicate that they have not yet been detected: DHAP, 5-hydroxy-4-aminohexaprenylphenol; DHHB, 3-hexaprenyl-4,5-dihydroxybenzoic acid; DHHP, 4,5-dihydroxy-hexaprenylphenol; HMHB, 3-hexaprenyl-4-hydroxy-5-methoxybenzoic acid; $IDDMQ_6H_2$, 4-amino-demethyl-demethoxyQ_6H_2.

digitonin-extracts of purified mitochondria. In such extracts the *O*-methyltransferase activity of Coq3 co-eluted with several of the other Coq polypeptides as high molecular mass complexes as determined by gel-filtration and by blue-native polyacrylamide gel electrophoresis (BN-PAGE) [18–21]. Indeed, the ability of Coq4 to organize high molecular mass complexes including Coq3 were shown to be essential for Q biosynthesis [19].

Several lines of evidence suggest that the Coq polypeptides and the multi-subunit Q-biosynthetic complex appear to be influenced by phosphorylation, either directly or indirectly due to Coq8. Coq8 (originally identified as Abc1) is a member of an ancient atypical kinase family [24]. Coq8/Abc1 homologs are required for Q biosynthesis in *E. coli* [25], yeast [26,27], and humans [28,29]. There is conservation of function as plant and human homologs of Coq8 are able to restore Q biosynthesis in yeast *coq8* mutants [30,31]. Conserved kinase motifs present in Coq8 are essential for maintenance of Q content [28,29,31], for the phosphorylation of Coq3, Coq5, and Coq7 polypeptides [21,31], and the association of Coq3 with a high molecular mass Coq polypeptide complex [21]. Collectively these studies suggest that maintenance or assembly of the Q-biosynthetic complex and phosphorylated forms of Coq3, Coq5, and Coq7 polypeptides depends on the presence of intact kinase motifs present in Coq8. However, it is important to note that kinase activity has not been demonstrated directly for yeast Coq8, or for the Coq8 homologs in prokaryotes, plants, or animals. Thus substrates of Coq8 have yet to be identified. In fact, there is evidence that phosphorylation may negatively regulate yeast Coq7 [32]. Moreover, recent work identified yeast Ptc7 as a mitochondrial phosphatase recognizing Coq7 and indicated that Ptc7 is required for optimal Q_6 content and function [33]. Thus although it appears that kinases and phosphatase activities modulate Q_6 biosynthesis and function in yeast, the role(s) played by Coq8 remain to be determined.

The content of Coq8 profoundly influences Q biosynthesis in *S. cerevisiae*. Over-expression of Coq8 was shown to restore synthesis of DMQ_6 in *coq7* null mutant yeast [17,34], suggesting the functional restoration of the Coq polypeptides up to this penultimate step of Q biosynthesis. In fact over-expression of Coq8 in the *coq3* and *coq5* null mutants restored steady-state levels of the Coq4, Coq6, Coq7, and Coq9 polypeptides [17,35]. Similarly, over-expression of Coq8 in the *coq3–coq9* null mutants restored steady-state levels of the unstable Coq polypeptides and resulted in the accumulation of late-stage Q-intermediates [17]. For example, over-expression of Coq8 in the *coq5* null mutant led to the synthesis of a late-stage Q intermediate diagnostic of the blocked *C*-methylation step (demethyl-demethoxy-Q_6, $DDMQ_6$) (Fig. 1) [17]. These results suggest a model whereby the over-expression of Coq8 stabilizes the remaining component Coq polypeptides, and allows the formation of high molecular mass Coq complexes.

A growing body of evidence indicates that Q or certain polyisoprenylated Q-intermediates also associate with the Q-biosynthetic complex. It was shown that DMQ_6 co-elutes with Coq3 *O*-methyltransferase activity and high molecular mass Coq polypeptide complexes during size exclusion chromatography of digitonin extracts of mitochondria [18]. Yeast *coq7* null mutants cultured in the presence of exogenous Q_6 were able to synthesize DMQ_6, and steady-state levels of Coq4 polypeptides were restored, indicating that the presence of Q_6 itself may stabilize the Coq polypeptide complexes [23,34]. Over-expression of Coq8 has no effect on either the *coq1* or *coq2* null mutants [17], which lack the ability to synthesize polyisoprenylated ring intermediates. This indicates that a polyisoprenylated component is essential for complex formation. Indeed, expression of diverse polyprenyl-diphosphate synthases, derived from prokaryotic species that do not synthesize Q, rescues Q synthesis in yeast *coq1* null mutants, and restores steady-state levels of the sensitive Coq polypeptides, including Coq4 and Coq6 [36]. Thus, exogenously supplied Q, or a polyisoprenylated Q-intermediate is postulated to interact with the complex and/or may stabilize certain of the Coq polypeptides.

Recent evidence suggests that the interaction between the Coq10 polypeptide and Q is essential for the function of Q in respiration and for efficient de novo synthesis of Q [37–39]. Respiration in mitochondria isolated from yeast *coq10* mutants can be rescued by the addition of Q_2, a soluble analog of Q_6. This is considered to be a hallmark phenotype of the yeast *coq* mutants unable to synthesize Q_6. However, unlike the *coq1–coq9* mutants, yeast *coq10* mutants retain the ability to synthesize Q_6, although its synthesis as measured with stable isotope-labeled ring precursors is less efficient [38]. The defects in Q respiratory function and de novo synthesis in the *coq10* mutant are rescued by human [37] or *Caulobacter crescentus* orthologs of Coq10 [38]. Structural determination of the *C. crescentus* Coq10 ortholog CC1736 identified a steroidogenic acute regulatory protein-related lipid transfer (START) domain [40]. The START domain forms a hydrophobic binding pocket and family members have been shown to bind sterols, phospholipids and other hydrophobic ligands. START domain proteins function as transporters and/or act as sensors of lipid ligands that regulate lipid metabolism and signaling [41,42]. The CC1736 START domain protein binds Q_{10}, Q_6, Q_3, Q_2 and DMQ_3, but not ergosterol or a farnesylated analog of HHB [38]. Thus, the Coq10 START polypeptide binds Q_n isoforms and facilitates both de novo Q biosynthesis and respiratory electron transport.

In this study we examine the sub-mitochondrial localization of the yeast Coq polypeptides, and determine the effects of over-expression of *COQ8* on the high molecular mass Coq polypeptide complexes in the *coq1–coq10* null mutants. The effects of Q supplementation on Coq polypeptide steady-state levels and the accumulation of Q-intermediates are also determined in each of the *coq* null mutants. The findings suggest that over-expression of Coq8 or Q_6 supplementation enhances the formation or maintenance of the Coq polypeptide complexes and are integrated into a new model of Q-biosynthesis.

2. Materials and methods

2.1. Yeast strains and plasmids

S. cerevisiae strains used in this study are listed in Table 1. Growth media for yeast were prepared as described [43], and included YPD (1% yeast extract, 2% peptone, 2% dextrose), YPGal (1% yeast extract, 2% peptone, 2% galactose, 0.1% dextrose) and YPEG (1% yeast extract,

Table 1
Genotype and source of yeast strains.

Strain	Genotype	Source
JM43	MAT α *leu2-3,112 ura3-52 trp1-289 his4-580*	[96]
W3031A	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
BY4741	MAT a *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
W303Δcoq1	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq1::LEU2*	[36]
W303Δcoq2	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq2::HIS3*	[97]
CC303	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq3::LEU2*	[98]
W303Δ*coq4*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq4::TRP1*	[99]
W303Δ*coq5*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq5::HIS3*	[45]
W303Δ*coq6*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq6::LEU2*	[49]
W303Δ*coq7*	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq7::LEU2*	[22]
W303Δ*coq8*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq8::HIS3*	[99]
BY4741Δ*coq9*	MAT a *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 coq9::KanMX4*	Open Biosystems
W303Δ*coq10*	MAT a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq10::HIS3*	[37]

[a] Dr. Rodney Rothstein, Department of Human Genetics, Columbia University.

2% peptone, 2% ethanol and 3% glycerol). Yeast were transformed with lithium acetate as described [44]. Transformed yeast strains were selected and maintained in SD-Ura (selective synthetic medium with 2% dextrose lacking uracil) [43], modified as described [45]. The p4HN4 plasmid used in this study (hcCOQ8) contains the *COQ8* gene in pRS426, a multi-copy yeast shuttle vector [46].

2.2. Mitochondrial isolation and immunoblot analyses with JM43 yeast

Yeast were cultured in YPGal medium (30 °C, 250 rpm) to an absorbance ($A_{600\ nm}$) of 2–4. Preparation of spheroplasts and fractionation of cell lysates were performed as described [47]. Crude mitochondria were isolated and further purified over a linear Nycodenz gradient as described previously [48]. Protein concentrations were determined with the bicinchoninic acid assay (Thermo). Indicated amounts of protein from the Nycodenz-purified mitochondrial fractions were analyzed by electrophoresis (SDS-PAGE) on 12% acrylamide, 2.5 M urea, Tris/glycine gels, then transferred to Hybond ECL Nitrocellulose (Amersham Biosciences). Subsequent immunoblot analyses and treatment of membranes for detection of antibodies were as described [49]. Primary antibodies to yeast mitochondrial polypeptides (Table 2) were used at the following concentrations: Coq1, 1:10,000; Coq2, 1:1000; Coq3, 1:1000; Coq4, 1:2000; Coq5, 1:5000; Coq6, 1:500; Coq7, 1:1000; affinity purified Coq8, 1:100; the beta subunit of F1-ATPase complex (Atp2), 1:4000; cytochrome b_2 ($Cytb_2$) 1:5000; cytochrome *c* (Cytc), 1:10,000; cytochrome c_1 ($Cytc_1$ 1:2000; and Heat shock protein 60 (Hsp60), 1:10,000. Goat anti-rabbit and goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (Calbiochem) were each used at a 1:10,000 dilution.

2.3. Sub-mitochondrial localization of Coq2 and Coq7 polypeptides

Mitochondria from JM43 yeast (3 mg protein, 150 μl) were suspended in five volumes of hypo-osmotic buffer (20 mM HEPES-KOH, pH 7.4), and incubated on ice for 30 min. The mixture was then centrifuged at 18,000 ×*g* for 20 min at 4 °C to separate the intermembrane space components (supernatant) from the mitoplasts (pellet), as described [50]. Mitoplasts were then sonicated (four 20-s-pulses on ice slurry, 20% duty cycle, 2.5 output setting; Sonifier W350, Branson Sonic Power Co.), then centrifuged at 100,000 ×*g* for 1 h at 4 °C to generate matrix (supernatant) and membrane (pellet) fractions. Alternatively, mitoplasts were subjected to alkaline carbonate extraction [51], and the mixture was then centrifuged at 100,000 ×*g* for 1 h at 4 °C to separate the integral membrane components (pellet) from the peripheral membrane and matrix components (supernatant). Equal aliquots of Nycodenz-purified mitochondria, untreated mitoplasts, pellet and supernatant fractions from either sonication or alkaline carbonate extraction, and intermembrane space components were subjected to SDS-PAGE analysis followed by immunoblot analyses.

Proteinase K treatment of mitochondria was performed as described [52] with some modifications. Proteinase K was added from a freshly made concentrated stock solution (10 mg/ml) to mitochondria suspended in buffer C (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4) (0.3 mg protein/ml) to a final concentration of 100 μg/ml. For treatment of mitoplasts, proteinase K was prepared in the hypo-osmotic buffer (20 mM HEPES-KOH, pH 7.4). When required, Triton-X100 or SDS were added to final concentration (w/v) of 1% or 0.5%, respectively, and incubated for 30 min at 4 °C. PMSF was added to inactivate the proteinase, followed by the addition of trichloroacetic acid (TCA; 60 °C) to a final concentration of 20%. The TCA pellets were subsequently collected by centrifugation and resuspended in Thorner buffer [53]; equal aliquots were processed for electrophoresis as described above.

Table 2
Description and source of antibodies.

Antibody	Source
Atp2	Carla. M. Koehler[a]
Coq1	[36]
Coq2	[20]
Coq3	[68]
Coq4	[67]
Coq5	[66]
Coq6	[49]
Coq7	[23]
Coq8	[20]
Coq9	[20]
Cytc	Carla M. Koehler[a]
$Cytb_2$	Carla M. Koehler[a]
$Cytc_1$	A. Tzagoloff[b]
Hsp60	Carla M. Koehler[a]
Mdh1	Lee McAlister-Henn[c]
Rip1	B. Trumpower[d]

[a] Dr. Carla. M. Koehler, Department of Chemistry and Biochemistry, UCLA.
[b] Dr. A. Tzagoloff, Department of Biological Sciences, Columbia University.
[c] Dr. Lee McAlister-Henn, Department of Molecular Biophysics and Biochemistry, University of Texas Health Sciences Center, San Antonio.
[d] Dr. B. Trumpower, Department Biochemistry, Dartmouth Medical School.

2.4. Salt-wash treatments of sonicated mitoplasts

Salt-wash treatments were performed as described previously [54] with some modifications. Equal volumes of sonication buffer (as a no salt control) or sonication buffer containing either KCl or NaCl were added to sonicated mitoplasts to final concentrations of 0.5 M or 1.0 M for KCl, and 0.5 M for NaCl. The samples were incubated on ice for 15 min, followed by centrifugation at 100,000 ×*g* for 1 h at 4 °C to separate the membrane associated components (pellet) from the soluble components (supernatant). Equal aliquots of starting mitochondria, unsonicated mitoplasts, intermembrane space components, membrane pellet, and supernatant fractions from salt-wash treatments of the sonicated mitoplasts were subjected to SDS-PAGE separation followed by immunoblot analyses.

2.5. Mitochondrial isolation and digitonin solubilization of W303 and BY4741 yeast strains

Yeast cultures were grown to an $A_{600\ nm}$ of 3–4 in YPGal media, and crude mitochondria were isolated from a total volume of 1.8 l of culture as described above. Crude mitochondria were further purified with an Optiprep discontinuous iodixanol gradient, and were collected from the interface of the gradient after ultracentrifugation. Briefly, the crude mitochondrial pellet was resuspended in 3 ml of Solution C. Solution C was prepared by adding 2 volumes of OptiPrep (60% w/v iodixanol; Sigma-Aldrich) to 1 volume of 0.8 M sorbitol, 60 mM HEPES-KOH, pH 7.4. Solutions of ρ (density) = 1.10 and 1.16 g/ml were prepared by mixing Solution C with Solution D (3 + 7 and 6.25 + 3.75, v/v respectively). Solution D contains 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4. In centrifuge tubes, 3 ml of crude mitochondria suspended in Solution C was layered at the bottom of a 14 × 89 mm Ultra-Clear centrifuge tube (Beckman), followed by 4.5 ml of the ρ = 1.16 g/ml iodixanol solution, and finally 4.5 ml of the ρ = 1.10 g/ml iodixanol solution was layered on top. Tubes were subjected to centrifugation (80,000 ×*g* for 3 h, 4 °C). The band of mitochondria was collected and washed with 10 volumes of solution D. Purified mitochondria were harvested by centrifugation at 12,000 ×*g* for 10 min, 4 °C, and were resuspended in 1 ml of solution D, and stored at −80 °C. Aliquots of purified mitochondria (200 μg) were solubilized in 50 μl of 1.6% digitonin, 1× protease inhibitor EDTA-free (Roche), 1:100 phosphatase inhibitor cocktail sets I and II (Calbiochem), 1× NativePAGE sample buffer (Invitrogen), and mitochondria suspension buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4). Samples were incubated on ice for

1 h with mixing by pipetting up and down every 20 min. The soluble supernatant fraction was separated from the insoluble pellet by centrifugation in a Beckman Airfuge (100,000 ×g, 10 min, chilled rotor).

2.6. Rescue of coq mutants with exogenous Q_6

Medium containing a final concentration of 10 μM Q_6 was prepared with a 6.54 mM Q_6 stock in ethanol; vehicle control medium contained an equivalent volume of added ethanol (1.5 μl/ml). Both Q_6-supplemented ($+Q_6$) and unsupplemented ($-Q_6$) YPD were sterile filtered. Designated wild-type W3031B or *coq* null mutants were grown in 20 ml YPD overnight and diluted to 0.1 $A_{600\ nm}$ in 18 ml of ($+Q_6$) or ($-Q_6$) YPD. Yeast cells were grown at 30 °C for 42 h. Cells (30 $A_{600\ nm}$) were centrifuged for lipid extraction and 145 pmol Q_4 was added to each cell pellet as an internal standard prior to lipid extraction. Yeast pellets were washed twice with distilled water before lipid extraction. Lipid extracts were analyzed by RP-HPLC-MS/MS [17]. Data were processed with Analyst version 1.4.2 software (Applied Biosystems). Cells (10 $A_{600\ nm}$) were collected by centrifugation for protein extraction as described [55]. Aliquots (corresponding to 1.3 $A_{600\ nm}$) of yeast whole cell lysates were separated by SDS-PAGE on 10% acrylamide gels followed by immunoblot analyses as described below.

To determine de novo synthesis of $^{13}C_6$-DMQ_6 in the *coq7* null mutant strain in the presence or absence of exogenous Q_6, cells were diluted to 0.1 $A_{600\ nm}$ in 18 ml of Q_6-supplemented ($+Q_6$) or unsupplemented ($-Q_6$) YPD. Media also contained 10 μg/ml $^{13}C_6$-4HB. Incubations proceeded for 42 h, and cell pellets were processed by lipid extraction and RP-HPLC-MS/MS as described above.

2.7. Two-dimensional Blue Native/SDS-PAGE and immunoblot analyses

Protein concentrations of purified mitochondria were determined by the bicinchoninic acid assay (Thermo). NativePAGE 5% G-250 sample additive (Invitrogen) was added to the supernatant from 200 μg of digitonin-solubilized mitochondria (50 μl) to a final concentration of 0.1%. BN-PAGE was performed as described in Native PAGE user manual with NativePAGE 4–16% Bis–Tris gel 1.0 mm × 10 wells (Invitrogen). First dimension gel slices were soaked in 65 °C 2× SDS sample buffer for 10 min before loading onto pre-cast 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P transfer membrane (Millipore), and blocked in 1% skim milk, phosphate-buffered saline, 0.1% Tween-20 (phosphate buffered saline is composed of 0.14 M NaCl, 1.2 mM NaH_2PO_4, and 8.1 mM Na_2HPO_4). Membranes were treated with the following primary antibodies (Table 2) at the dilution indicated: Coq4, 1:250; Coq7, 1:1000; Coq9, 1:1000; porin, 1:1000. Secondary antibodies were goat anti-rabbit IgG H&L chain specific peroxidase conjugate (Calbiochem).

3. Results

3.1. Sub-mitochondrial localization of Coq2 and Coq7 polypeptides

According to earlier studies [50,56], the yeast Coq2 and Coq7 proteins both reside in mitochondria. However, the sub-mitochondrial localization of these proteins (in their untagged forms) was not determined. To determine the sub-mitochondrial localizations of Coq2 and Coq7, yeast mitochondria were further fractionated as described in Materials and methods. Purified mitochondria were treated with hypotonic buffer, resulting in the disruption of the outer membrane and subsequent release of soluble components of the intermembrane space while keeping the inner membrane intact. Immunoblot analyses of the sub-mitochondrial fractions indicated that Coq2 and Coq7 polypeptides associated with the pellet (mitoplast fraction) and did not co-localize with cytochrome b_2 (Cytb2), the intermembrane space marker (Fig. 2A).

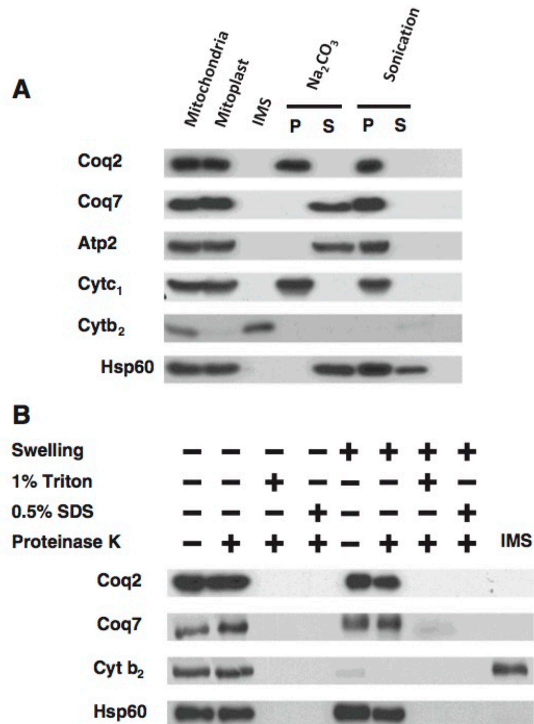

Fig. 2. Coq2 is an integral membrane protein while Coq7 is peripherally associated to the inner mitochondrial membrane, facing toward the matrix side. (*A*), Mitochondria were subjected to a hypotonic swelling and centrifugation to separate intermembrane space protein (*IMS*) and mitoplasts. The mitoplasts were treated with 0.1 M Na_2CO_3, pH 11.5, or sonicated, then separated by centrifugation (100,000 ×g for 1 h) into supernatant (*S*) or pellet (*P*) fractions. (*B*), Intact mitochondria or mitoplasts were treated with 100 μg/ml Proteinase K for 30 min on ice, with or without detergent. Equal aliquots of pellet and TCA-precipitated soluble fractions were analyzed. Mitochondrial control markers are: Atp2, peripheral inner membrane protein; Cytb2, inter-membrane space protein; $Cytc_1$, integral inner membrane protein; and Hsp60, soluble matrix protein.

Mitoplasts were further fractionated either by sonication, releasing soluble matrix components into the supernatant following high speed centrifugation, or by extraction with alkaline carbonate, which releases peripherally bound membrane proteins into the supernatant [57]. Sonication treatment partially dissociated Hsp60, the matrix marker [58], however, neither Coq2 nor Coq7 was released from the membrane/pellet fraction (Fig. 2A). Coq7 was released into the supernatant by alkaline carbonate extraction in a manner similar to Atp2, a peripheral inner membrane protein [59], while Coq2 remained in the pellet, along with $Cytc_1$ an integral membrane marker [60]. These results indicated that Coq2 is an integral membrane protein while Coq7 behaves as a peripheral membrane protein.

To further characterize the membrane association of Coq2 and Coq7 proteins, purified mitochondria or mitoplasts were treated with Proteinase K in the absence and presence of detergent (1% Triton X-100 or 0.5% SDS). The results (Fig. 2B) showed that Coq2, Coq7, and Hsp60 polypeptides were protected from the protease both in intact mitochondria and in mitoplasts. As expected, detergent treatment of either mitochondria or mitoplasts rendered all proteins protease-sensitive. The results indicate that both Coq2 and Coq7 polypeptides are inner membrane proteins facing the matrix side in yeast mitochondria.

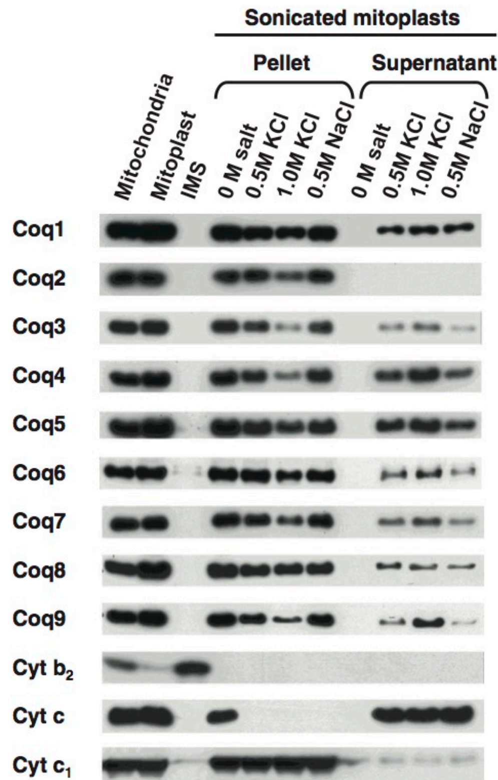

Fig. 3. Coq2 is resistant to salt extraction of sonicated mitoplasts while other Coq proteins are partially disassociated from the mitochondrial inner membrane. Purified mitochondria were subjected to hypotonic swelling to generate mitoplasts. Equal volumes of sonication buffer with or without salt were added to sonicated mitoplasts. Samples were incubated for 15 min on ice then separated by centrifugation (100,000 ×g for 1 h) into supernatant or pellet fractions. Equal aliquots of pellet and TCA-precipitated supernatant fractions were analyzed.

3.2. Coq2 is resistant to salt extraction while other Coq polypeptides are sensitive

The above sub-mitochondrial localization results indicated that Coq7 is a peripheral membrane protein. However, modeling studies have predicted Coq7 to be an interfacial inner mitochondrial membrane protein [61,62]. Interfacial membrane proteins, such as prostaglandin synthase [63] and squalene cyclase [64], are embedded in the membrane via interaction with only one leaflet of the bilayer. To distinguish between a peripheral and an interfacial membrane association for Coq7, salt extraction analyses (with 0.5 M KCl, 1.0 M KCl, or 0.5 M NaCl) were performed on sonicated mitoplasts prepared as described above. The resulting mixtures were subsequently separated into supernatant and pellet (membrane associated) fractions via high-speed centrifugation. Western blot analysis of the fractions (Fig. 3) showed that Coq7 and each of the Coq polypeptides, except for Coq2, were partially released from the membrane following the addition of salt. Interestingly, the degree of dissociation of these proteins depended on salt concentration and not on its identity per se, KCl versus NaCl. In contrast, Coq2 and the integral membrane marker, Cytc$_1$, were resistant to salt extraction and thus remained in the membrane fraction. Cytochrome *c*, which peripherally attaches to the inner mitochondrial membrane through electrostatic interactions with fatty acids and acidic phospholipids [65], was released from the sonicated mitoplasts following salt addition, as expected. These results provide further support for the sub-mitochondrial localization data indicating that yeast Coq7 is a peripheral membrane protein on the matrix side as are Coq1, Coq3, Coq4, Coq5, Coq6, Coq8, and Coq9 polypeptides [20,31,36,49,66–68].

3.3. The Coq4 and Coq9 polypeptides are sensitive indicators of the Coq polypeptide Q-biosynthetic complexes — and over-expression of COQ8 stabilizes these complexes in certain coq null mutants

The co-localization of the Coq polypeptides with the mitochondrial inner membrane is consistent with their interaction in Q-biosynthetic complexes. The yeast Coq4 and Coq9 polypeptides co-purify with other Coq polypeptides and both migrate at high molecular mass in separation of digitonin extracts of mitochondria [18–21]. Thus, we used the Coq4 and Coq9 polypeptides as sensitive indicators of the state of the high molecular mass Coq complexes. Mitochondria from wild type and *coq* null mutant yeast were purified, solubilized with digitonin, separated by two-dimensional blue native/SDS PAGE, and antibodies against Coq4 and Coq9 were used to detect their presence. In digitonin extracts of wild-type mitochondria, Coq4 and Coq9 polypeptides are detected in several high molecular mass complexes (from 669 to >880 kDa) (Fig. 4). The Coq4 and Coq9 polypeptides are also detected at lower molecular mass (66–440 kDa), perhaps indicating their presence in partial- or distinct sub-complexes. In contrast, Coq4 and Coq9 were not detected in digitonin extracts of mitochondria isolated from *coq3*, *coq4*, *coq5*, or *coq7* null mutant strains (Figs. 4 and 5). In each of the *coq3–coq9* null mutant strains, the lack of one of the Coq polypeptides is thought to destabilize the Coq polypeptide complex, and the mutants accumulate only the early Q-intermediates HHB and HAB, generated from the aromatic ring precursors 4HB and pABA, respectively (Figs. 1, 4 and 5). In contrast, the Coq4 and Coq9 polypeptides are detected at high molecular mass in the *coq6* null mutant, and the Coq4 polypeptide is detected in the *coq9* null mutant (Fig. 5). These observations are consistent with the presence of steady state levels of these polypeptides noted previously in these two null mutants [17]. Schematics showing possible interactions between the Coq polypeptides are depicted in Figs. 4 and 5.

The over-expression of Coq8, a putative kinase, has dramatic effects on the phenotypes of the *coq* null mutants. Over-expression of Coq8 restores steady state levels of several of the Coq polypeptides, and enables the synthesis of late-stage Q-intermediates in several of the *coq* null mutants [17]. To investigate whether over-expression of Coq8 stabilizes high molecular mass Coq polypeptide complexes, mitochondria were prepared from *coq* null mutant yeast over-expressing Coq8, and digitonin extracts were separated by two-dimensional blue native/SDS PAGE. We were particularly interested in examining the high molecular mass complexes in the *coq3* and *coq4* mutants, because over-expression of Coq8 stabilizes the Coq6, Coq7, and Coq9 polypeptides, yet the *coq3* and *coq4* mutants persist in accumulating early Q-intermediates. Over-expression of Coq8 in the *coq3* mutant restored Coq4 and Coq9 polypeptides to both high and low molecular mass complexes (440–880 kDa and 66 kDa) (Fig. 4), yet only early-stage intermediates HHB (with 4-HB as precursor) and HAB (with pABA as precursor) were detected in this strain [17]. Over-expression of Coq8 in the *coq4* null mutant restored the presence of the Coq9 polypeptide, although it was detected in only a low molecular mass complex (66 kDa) (Fig. 4); under these conditions, HHAB is detected (Fig. 1) [17], indicating the presence of functional Coq6. These results indicate that although over-expression of Coq8 stabilizes Coq6, Coq7 and Coq9 polypeptides in the *coq4* mutant, in the absence of the Coq4 polypeptide, a high molecular mass complex is not observed, and HHAB is the only novel Q-intermediate detected. Conversely, although the over-expression of Coq8 in the *coq3* mutant restores high molecular mass complexes of Coq4 and Coq9, this does not appear to result in production of new Q-intermediates.

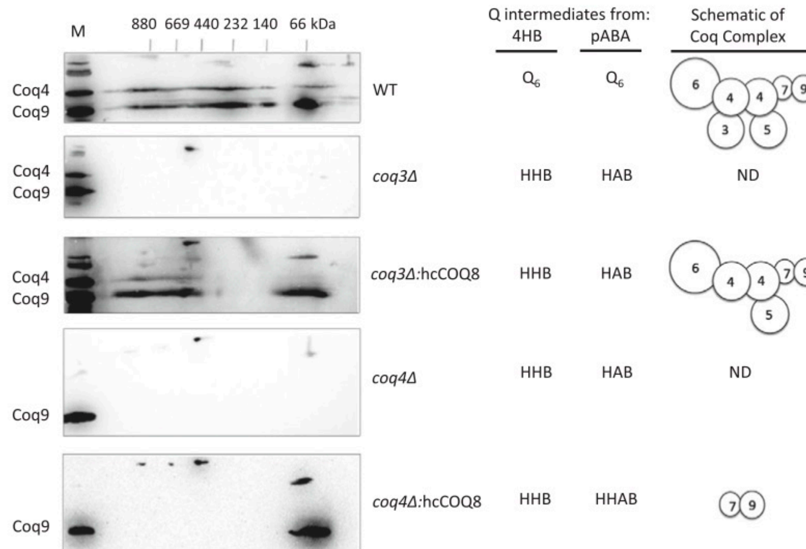

Fig. 4. Over-expression of Coq8 in the *coq3* null mutant, but not in the *coq4* null mutant, stabilizes the multi-subunit Coq polypeptide complex. Mitochondria were isolated from WT (W303-1A), *coq3* null or *coq4* null with and without the over-expression of Coq8 (hcCOQ8). Purified mitochondria (200 μg protein), were separated by two-dimensional blue native/ SDS PAGE, and the immunoblots were probed with antibodies against Coq4 and Coq9. A sample of wild-type mitochondria separated just in the SDS-second dimension served as a positive control and is designated by *M*. Q or Q-intermediates derived from either 4HB or pABA that accumulated in the yeast strains were determined in the study by Xie et al. [17]. The *coq* mutants over-expressing Coq8 continue to produce HHB and HAB, but in addition the *coq4* mutant also accumulates HHAB. Schematics show interactions of the Coq polypeptides and illustrate interactions potentially favored by over-expression of Coq8; ND, Coq polypeptides not detected.

The effects of Coq8 over-expression on the native molecular mass of the Coq4 and Coq9 polypeptides were also studied in the *coq5*, *coq6*, *coq7*, and *coq9* null mutants. Over-expression of Coq8 restored the Coq4 and Coq9 polypeptides to several high and low molecular mass complexes in the *coq5* and *coq6* null mutants (Fig. 5). Over-expression of *COQ8* restored the Coq4 polypeptide to a high molecular mass complex and the Coq9 polypeptide to a low molecular mass complex in the *coq7* null yeast mutant (Fig. 5). In the *coq5*, *coq6* and *coq7* null mutants, over-expression of Coq8 enables synthesis of late-stage Q-intermediates: *coq5* null mutant accumulates DDMQ$_6$, *coq6* null accumulates 4-HP (with 4-HB as precursor) and 4-AP (with pABA as precursor), and *coq7* null accumulates DMQ$_6$ [17]. Coq4 steady-state levels decrease dramatically in the *coq9* null mutant, but a small amount of Coq4 is detected near 669 kDa. Over-expression of *COQ8* in the *coq9* null mutant has only mild effects on Coq4 steady-state levels [17], but Coq4 is present at a higher molecular weight (around 800 kDa) (Fig. 5). The *coq9* null mutant harboring multi-copy Coq8 accumulates 4-HP and DMQ$_6$ (with 4-HB as precursor) and 4-AP and IDMQ$_6$ (with pABA as precursor) [17]. These results indicate that over-expression of *COQ8* stabilizes Coq polypeptide complexes in several of the *coq* null mutants.

3.4. Over-expression of COQ8 enhances Coq4 and Coq9 levels in a coq10 null mutant

Previous work showed that steady-state levels of Coq4, Coq6, Coq7, and Coq9 polypeptides were decreased in the *coq10* null mutant [20]. Although the *coq10* null mutant produces Q$_6$, the rate of de novo Q$_6$ biosynthesis is decreased relative to that of wild-type yeast [38]. Moreover, the respiratory defect and Q$_6$ de novo biosynthesis in the *coq10* mutant is rescued by over-expression of *COQ8* [37,38]. Over-expression of *COQ8* enhances steady-state levels of Coq4 and Coq9 in the *coq10* null mutant (Fig. 6A). While both Coq4 and Coq9 are detected in high molecular mass complexes in the *coq10* null mutant, over-expression of *COQ8* appears to increase the association of Coq4 with the complex (Fig. 6B).

3.5. Q$_6$ supplementation changes steady-state levels of certain Coq polypeptides and promotes accumulation of late-stage Q-intermediates in certain coq null mutants

Exogenous Q$_6$ has been shown to rescue the growth of the *S. cerevisiae coq2*, *coq3*, *coq5*, *coq7*, *coq8*, *coq9*, and *coq10* null mutants on media containing non-fermentable carbon sources [12,26,37,50,69]. We were able to rescue the growth of each of the *coq1*–*coq9* null mutants on YPEG medium containing ethanol and glycerol as non-fermentable carbon sources, with the addition of 2 μM Q$_6$ to the medium (data not shown). To determine the effect of exogenous Q$_6$ on the Coq polypeptide levels, each of the *coq1*–*coq9* null mutants was cultured in YPD in the presence or absence of exogenous Q$_6$. For these experiments YPD medium was chosen because growth of the *coq* null mutants in the absence of Q$_6$ is supported by dextrose. Previous studies indicate that both plasma membrane and mitochondrial Q$_6$ content in *coq7* null mutant (W303 genetic background) were increased when cultured in YPD supplemented with 2 μM Q$_6$ [70]. Succinate-cytochrome *c* reductase activity also increased under these conditions, indicating exogenous Q$_6$ restored activity in the mitochondrial respiratory chain [70]. The YPD medium was supplemented with 10 μM Q$_6$, because this concentration is near optimal for restoration of growth [69]. Wild-type yeast and each of the *coq* null mutants in YPD were cultured in either the presence or absence of 10 μM Q$_6$. The addition of Q$_6$ does not appear to affect mitochondrial protein levels in wild-type yeast (Fig. 7). However, Q$_6$ supplementation increases Coq9 polypeptide steady-state levels in *coq3*, *coq4*, *coq6* and *coq7* null mutants and increases Coq4 in *coq3*, *coq6* and *coq7* null mutants (Fig. 7). The most significant increases in Coq4, Coq7 and Coq9 polypeptide levels were observed in the *coq6* null mutant supplemented with Q$_6$ (Fig. 7). In contrast, Q$_6$ supplementation decreases steady-state levels of Coq1 in *coq2*–*coq9* null mutants. To determine whether

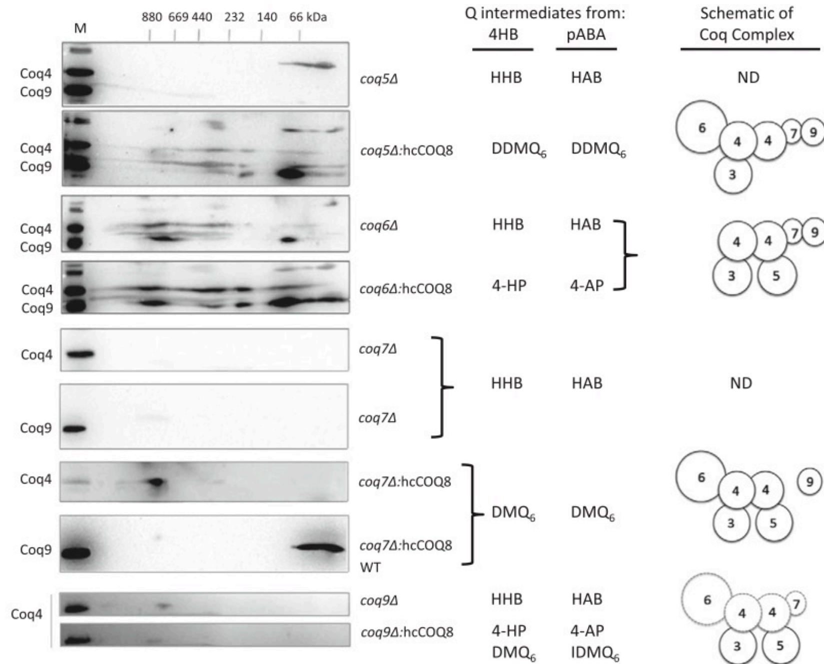

Fig. 5. Over-expression of Coq8 in *coq5*, *coq6*, *coq7* or *coq9* null mutant strains stabilizes the multi-subunit Coq polypeptide complex. Mitochondria were isolated from yeast strains harboring a deletion in one of the *coq5*, *coq6*, *coq7*, or *coq9* genes with and without the over-expression of Coq8 (hcCOQ8). Purified mitochondria (200 μg protein) were separated by two-dimensional blue native/SDS PAGE, and the immunoblots were probed with antibodies against Coq4 and Coq9. A sample of wild-type mitochondria separated only in the SDS-second dimension served as a positive control and is designated by *M*. Q-intermediates derived from either 4HB or pABA that accumulated in the yeast mutants were determined in the study by Xie et al. [17]. The *coq* mutants over-expressing Coq8 continue to produce HHB and HAB, but in addition the designated late-stage Q-intermediates are also observed. Schematics show interactions of the Coq polypeptides and illustrate interactions potentially favored by over-expression of Coq8; *dotted lines* indicate that steady state-Coq polypeptides are present but are decreased relative to wild type; ND, Coq polypeptides not detected.

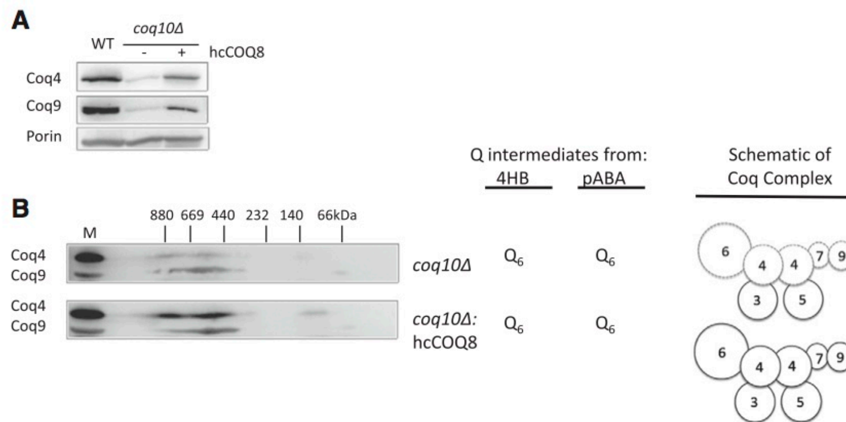

Fig. 6. Over-expression of Coq8 in *coq10* null mutant strain stabilizes the Coq4 and Coq9 polypeptide levels. Mitochondria were purified from *coq10* null mutant yeast strain with and without the over-expression of Coq8 (hcCOQ8). (*A*), Purified mitochondria (20 μg protein) were subject to SDS-PAGE and Western blot probing with antibodies against Coq4, Coq9, and Porin. (*B*), Purified mitochondria (200 μg protein), were subjected to two-dimensional Blue Native/SDS PAGE, and immunoblots were probed with antibodies against Coq4 and Coq9. A sample of wild-type mitochondria separated only in the SDS-second dimension served as a positive control and is designated by *M*. The *coq10* mutant produces Q_6 from 4HB and pABA and retains high molecular mass complexes of the Coq polypeptides as indicated by the schematic of the Coq complex; *dotted lines* indicate that steady state-Coq polypeptides are present but are decreased relative to wild type.

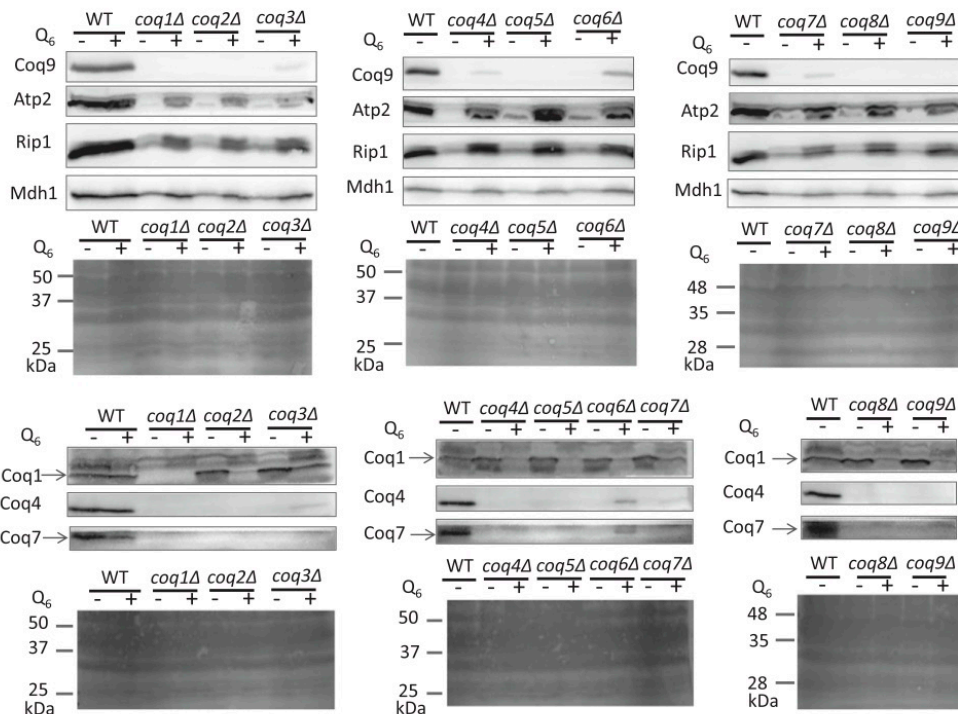

Fig. 7. Inclusion of exogenous Q_6 during culture of *coq1–coq9* null yeast mutants stabilizes certain Coq and mitochondrial polypeptides. Wild type or *coq1–coq9* null mutant yeast were grown in 18 ml of YPD with either 1.5 μl ethanol/ml medium (no Q_6 addition) or the same volume of Q_6 dissolved in ethanol giving a final concentration of 10 μM Q_6 (+Q_6) for 42 h. Yeast cells (10 $A_{600\ nm}$) were collected as pellets. Protein extracts were prepared from the pellets and analyzed by SDS-PAGE and immunoblot. Immunoblots were performed with antibodies against Coq1, Coq4, Coq7, Coq9, Atp2, malate dehydrogenase (Mdh1), or Rieske iron–sulfur protein (Rip1). Ponceau staining was used to detect the total proteins transferred to the membrane and served as the loading control.

supplementation with Q_6 affects other mitochondrial proteins, we investigated the steady-state levels of the beta subunit of the F1 sector of mitochondrial F_1F_0 ATP synthase (Atp2), malate dehydrogenase (Mdh1), and the Rieske iron–sulfur protein (Rip1) of the cytochrome bc_1 complex. The addition of Q_6 increases steady-state levels of Atp2, Mdh1 and Rip1 in each of the *coq* null mutants, suggesting that supplementation with Q_6 may have general protective effects on mitochondria.

The effect of Q_6 supplementation on Q intermediates was assessed in each of the *coq* null mutants. The *coq3–coq9* null mutants accumulate only early stage intermediates HHB and HAB (Fig. 1). However, DMQ_6 is produced in *coq7* null mutants cultured in the presence of exogenous Q_6 [34]. Here, we used HPLC with tandem mass spectrometry to detect Q_6 intermediates in lipid extracts of *coq* null mutants cultured in either the presence or absence of 10 μM exogenous Q_6. We confirmed the accumulation of DMQ_6 in the *coq7* null mutant cultured in exogenous Q_6 (Fig. 8). Since exogenous Q_6 contains a small amount of DMQ_6, $^{13}C_6$-4HB was used to detect de novo synthesis of $^{13}C_6$-DMQ_6. A very small amount of $^{13}C_6$-DMQ_6 was detected in *coq7* null mutant labeled with $^{13}C_6$-4HB. (We note that DMQ_6 is detectable in *coq7* null mutant when lipid extracts are prepared from 30 $A_{600\ nm}$ or more yeast and attribute this to the high sensitivity LC-MS/MS system.) In the presence of 10 μM exogenous Q_6, $^{13}C_6$-DMQ_6 accumulation increased significantly in *coq7* null mutant labeled with $^{13}C_6$-4HB. $^{13}C_6$-DMQ_6 was identified by its retention time of 4.56 min (the same as DMQ_6), and a precursor-to-product ion transition of 567.0/173.0, consistent with the presence of the $^{13}C_6$-ring (Fig. 8).

In addition, exogenous Q_6 led to an increased accumulation of 3-hexaprenyl-4-amino-5-hydroxybenzoic acid (HHAB) in *coq4* (Fig. 9A). This intermediate has a retention time of 2.69 min and a precursor-to-product ion transition of 562.0/166.0 detected with multiple reaction monitoring (MRM). We have previously detected HHAB in lipid extracts of *coq4-1* mutants, harboring a point mutation [17]. Surprisingly, smaller but readily detectable amounts of HHAB (a product of the Coq6 step) were also detected in the *coq6* mutant cultured with exogenous Q_6 (Fig. 9B). In addition to HHAB, 4-AP increased significantly in the *coq6* null mutant cultured with exogenous Q_6 (Fig. 10A). 4-AP was identified by its retention time (2.88 min), precursor-to-product ion transition (518.5/162.2), and fragmentation spectrum (Fig. 10B). 4-AP has been shown to accumulate in certain *coq6* point mutants [16], and in *coq6* and *coq9* null mutants over-expressing Coq8 [17]. The addition of Q_6 caused the accumulation of imino-demethoxy-Q_6 ($IDMQ_6$) in the *coq9* null mutant (Fig. 11A). This intermediate has a retention time of 4.9 min and a precursor-to-product ion transition of 560.5/166.1. Its identity is further confirmed by the fragmentation spectrum (Fig. 11B). In contrast, late-stage Q-intermediates were not detected in the other *coq* null mutants. Thus only *coq4*, *coq6*, *coq7* and *coq9* null mutants accumulate late-stage Q-intermediates upon the addition of Q_6. These data indicate that Q_6 stabilizes the Q-biosynthetic complex and allows later Q-intermediates to accumulate.

4. Discussion

This study examined the location and organization of the yeast mitochondrial Q-biosynthetic complex. We found that over-expression of Coq8, an ancient atypical putative kinase, stabilizes the high molecular mass Coq polypeptide complex in several of the *coq* null mutants.

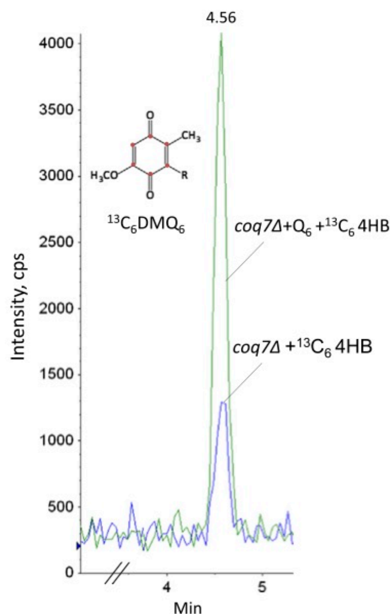

Fig. 8. Exogenous Q_6 increases synthesis of demethoxy-Q_6 (DMQ_6) in *coq7* null mutant. Yeast *coq7* null mutant was cultured in YPD with either 10 μg/ml $^{13}C_6$-4HB and 1.5 μl ethanol/ml medium (no Q_6 addition) or 10 μg/ml $^{13}C_6$-4HB and the same volume of Q_6 dissolved in ethanol giving a final concentration of 10 μM Q_6 (+Q_6) for 42 h. Yeast cells (30 $A_{600\ nm}$) were collected as pellets and washed twice with distilled water. Q_4 (145.4 pmol) was added as internal standard. Lipid extracts were prepared from the pellets and analyzed by RP-HPLC-MS/MS. Multiple reaction monitoring (MRM) detected precursor-to-product ion transitions 567.0/173.0 ($^{13}C_6$-DMQ_6). The *green trace* designates the $^{13}C_6$-DMQ_6 signal in the + Q_6 condition, and the *blue trace* indicates the $^{13}C_6$-DMQ_6 signal in the absence of added Q_6. The peak areas of $^{13}C_6$-DMQ_6 normalized by peak areas of Q_4 are 0.0665 in *coq7Δ* + $^{13}C_6$-4HB and 0.215 in *coq7Δ* + Q_6 + $^{13}C_6$-4HB.

Supplementation of growth medium with exogenous Q_6 restored steady-state levels of Coq polypeptides and enhanced the production of late-stage Q-intermediates in certain *coq* null mutants. Based on the observed interdependence of the Coq polypeptides, the effect of exogenous Q_6, and the requirement for an endogenously produced polyisoprenyl intermediate (summarized in Table 3), we propose a new model for the CoQ-synthome, a Coq multi-subunit polypeptide and lipid complex required for the biosynthesis of Q in yeast (Fig. 12).

The Coq4 and Coq9 polypeptides were used as sensitive indicator polypeptides to monitor the state of high molecular mass Coq polypeptide complexes in digitonin extracts of mitochondria, as assayed by two-dimensional blue native/SDS PAGE. The over-expression of Coq8 in the *coq* null mutants was found to profoundly affect the association of Coq4 and Coq9 in high molecular mass complexes. The Coq4 polypeptide persists at high molecular mass with the over-expression of Coq8 in the *coq3*, *coq5*, *coq6*, *coq7*, and *coq9* null mutants (Figs. 4 and 5). This finding indicates that deletion of any of these Coq polypeptide components has little impact on the association of Coq4 with a high molecular mass Coq complex. The Coq9 polypeptide persists at high molecular mass with the over-expression of Coq8 in the *coq3*, *coq5* and *coq6* null mutants, but is present only at low molecular mass in the *coq4* and *coq7* null mutants upon Coq8 over-expression (Fig. 5). Hence, we propose that Coq4 may be a crucial component through which Coq3, Coq5, Coq6, Coq7, and Coq9 associate to form the CoQ-synthome. Coq7 is an important component through which Coq9 associates with Coq4.

Based on these findings our model depicts Coq4 as a central organizer, and the Coq3, Coq5 and Coq6 polypeptides as more peripheral members of the CoQ-synthome (Fig. 12). In this model Coq4 is depicted as a homodimer, with each monomer harboring a binding site for the polyisoprenyl-tail of Q_6 or a polyisoprenyl-intermediate. This is based on the structure determined for Alr8543, a Coq4 homolog from *Nostoc* sp. *PCC7120*, and the molecular modeling of the highly similar *S. cerevisiae* Coq4 [71]. Each monomer of the Alr8543 homodimer co-crystalized with a geranylgeranyl monophosphate, and Rea et al. [71] proposed that yeast Coq4 may similarly bind to the polyisoprenyl tail of HHB (or HAB), consistent with the idea that Coq4 forms a scaffold organizing the Coq polypeptide complex [19], facilitating the action of the Coq6 hydroxylase, the Coq3 and Coq5 methyltransferases, and the Coq7 hydroxylase. The model is also consistent with the hypothetical branched biosynthetic scheme of Q biosynthesis (Fig. 1). For example, in the presence of hcCOQ8, *coq6* or *coq9* mutants accumulate 4-AP (derived from pABA), and 4-HP (derived from 4HB) [17], indicating that in some cases decarboxylation and hydroxylation at position 1 of the ring (catalyzed by yet to be identified enzymes) might precede the Coq6 hydroxylation step.

The CoQ-synthome represents a minimal schematic model because the total predicted mass based on the sum of the component Coq polypeptides is only 230–240 kDa [31] (Fig. 12); this is well below the 1 MDa size of the complex estimated from blue-native gels. The stoichiometry of the Coq polypeptides in the complex is not known and it is likely that additional components remain to be identified. The model is consistent with the peripheral association of each of the Coq polypeptides to the matrix side of the mitochondrial inner membrane, with the exception of Coq2 (Figs. 2 and 3). In addition to interaction with Coq4, it is possible that the association of Coq polypeptides with the inner mitochondrial membrane may derive from interactions with Q_6 and/or a polyisoprenyl-intermediate. So far, Coq2 is the only integral membrane protein of the Q-biosynthetic proteins. Previous models suggested that Coq2 might serve as an ideal anchor-protein candidate for the Coq complex [12], and blue native/SDS PAGE indicated Coq2 migrated at high molecular mass [21]. However, co-precipitation experiments have so far failed to identify any physical interactions between Coq2 and the other Coq polypeptides (data not shown). Based on this, Fig. 12 shows Coq1 and Coq2 independently generate HHB or HAB, early Q-intermediates that accumulate in each of the *coq3*–*coq9* null mutants.

Studies in *S. cerevisiae* and *Schizosaccharomyces pombe* have set the stage for understanding Q biosynthesis in animals; many human and mouse COQ homologs have been shown to rescue the corresponding yeast *coq* mutants [14,72]. Expression of human COQ4 has been shown to rescue the *S. cerevisiae coq4* null mutant [73], suggesting that human COQ4 might maintain interactions with yeast Coq polypeptides. However, certain animal Q biosynthetic proteins require specific partner proteins to observe cross complementation of the yeast mutant. For example, Pdss1 and Pdss2 (Coq1 homologs) from *S. pombe*, mouse, and human form heterotetrameric complexes, and must be co-expressed to reconstitute synthesis of the polyisoprene-diphosphate tail [74,75]. Human COQ9 has not yet demonstrated interspecific complementation of the yeast *coq9* mutants [76]; this might be due to interactions of Coq9 with Coq7. Similar to yeast, the function of Coq7 in mouse requires Coq9. A homozygous $Coq9^{X/X}$ mouse, containing a Coq9-R239X stop codon mutation, displayed a severe reduction of Q_9 content, accumulated DMQ_9, and showed a profound decrease in steady state Coq7 polypeptide levels [77]. The $Coq9^{X/X}$ mouse model was patterned after human patients with Q deficiency and mitochondrial encephalomyopathy [76]. The recapitulation of the human disease in the $Coq9^{X/X}$ mouse model suggests that COQ7 hydroxylation of DMQ requires COQ9 in mice and humans. Other interactions between human COQ polypeptides have been reported recently. ADCK4 (a human homolog of yeast Coq8) was shown to interact with COQ6 and COQ7 polypeptides in podocyte cell cultures [78]. Although we have not detected Coq8 in direct association with any of the yeast Coq polypeptides, it is tempting to speculate that human ADCK4 may

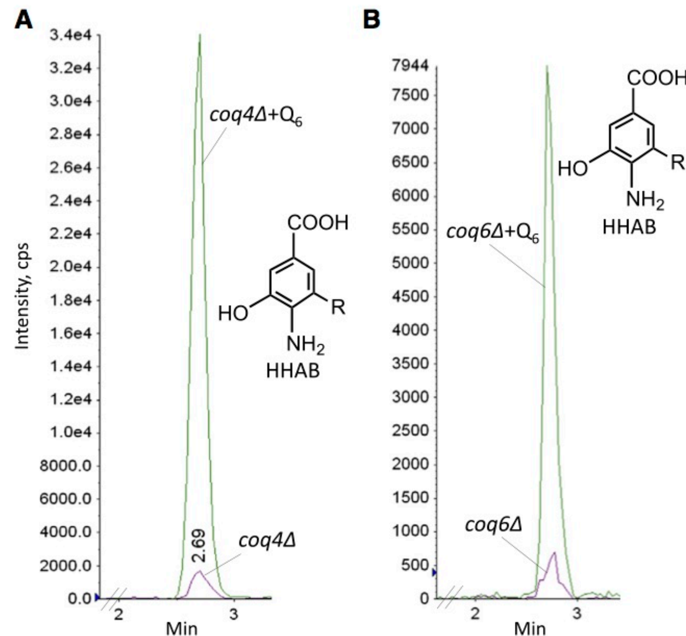

Fig. 9. Exogenous Q_6 increases the accumulation of 3-hexaprenyl-4-amino-5-hydroxybenzoic acid (HHAB) in *coq4* and *coq6* null mutants. Yeast *coq4* and *coq6* null mutants were cultured in YPD with either 1.5 μl ethanol/ml medium (no Q_6 addition) or the same volume of Q_6 dissolved in ethanol giving a final concentration of 10 μM Q_6 ($+Q_6$) for 42 h. Yeast cells (30 $A_{600\ nm}$) were collected as pellets and washed twice with distilled water. Q_4 (145.4 pmol) was added as internal standard. Lipid extracts were prepared from the pellets and analyzed by RP-HPLC-MS/MS. Multiple reaction monitoring (MRM) detected precursor-to-product ion transitions 562.0/166.0 (HHAB) and 455.4/197.0 (Q_4). The arbitrary units (cps) and the scale is the same for all the traces within the same panel. In panels *A* and *B*, green traces designate the HHAB signal in the $+Q_6$ condition, and purple traces the HHAB signal in the absence of added Q_6. The peak areas of HHAB normalized by peak areas of Q_4 are 0.01 in *coq4Δ* (A), 0.14 in *coq4Δ* + Q_6 (A), 0.003 in *coq6Δ* (B), and 0.03 in *coq6Δ* + Q_6 (B). The retention times of HHAB are 2.69 min in *coq4Δ* + Q_6 (A), and 2.71 min in *coq6Δ* + Q_6 (B).

recognize COQ6 and COQ7 as potential substrates for phosphorylation. Interestingly, while expression of ADCK4 failed to rescue the yeast *coq8* mutant [78], expression of human ADCK3 did rescue the *coq8* mutant, partially restore Q_6 content as well as phosphorylated forms of yeast Coq3, Coq5, and Coq7 [31].

We investigated the effects of Coq8 over-expression on the Coq4 and Coq9 polypeptides in the *coq10* null mutant. Over-expression of Coq8 in the *coq10* null mutant increases steady-state levels of the Coq4 and Coq9 polypeptides and their association with the high molecular mass Coq complexes (Fig. 6). In the *coq10* null mutant the rate of Q biosynthesis is reduced but may be significantly increased by Coq8 over-expression, or by the expression of a START domain ortholog of Coq10 [38]. These findings are consistent with the model that the Coq10:Q polypeptide ligand complex functions as a chaperone of Q and that Q delivery to the CoQ-synthome is necessary for efficient de novo Q biosynthesis (Fig. 12), and/or for delivery of Q to the N-site of the bc_1 complex [38,79]. It is tempting to speculate that Coq10 may function to chaperone the "inactive" pool of Q (depicted as residing at the midplane of the bilayer [80,81]) to form an "active" pool of Q, consistent with a dedicated subset of Q molecules performing electron transport within the respirasomes [82,83].

Results presented here show that exogenous Q_6 restores the growth of any of the *coq1*–*coq10* null mutant yeast in medium containing a nonfermentable carbon source. This effect of supplementation with exogenous Q_6 is known to require uptake; Q_6 binds to soluble proteins derived from peptone in the growth medium and is taken up by cells and transported to mitochondria via an endocytic pathway [84]. James et al. [85] identified 16 yeast *ORFs* required for utilization of exogenous Q_4 in a yeast double knockout library (*ΔORFΔcoq2*). We determined the steady-state levels of the Coq4, Coq7, and Coq9 polypeptides as indicators of the CoQ-synthome, and scanned for Q_6-intermediates by HPLC tandem mass spectrometry. Upon the addition of Q_6, the *coq3*, *coq4*, *coq6*, *coq7* and *coq9* null mutants accumulate distinct hexaprenyl Q-intermediates and/or show increased steady-state levels of one or more of the indicator Coq polypeptides (Figs. 8–11 and Table 3). These findings confirm and extend previous studies showing that addition of exogenous Q_6 restored de novo synthesis of DMQ_6 and increased steady-state levels of the Coq4 polypeptide in a *coq7* null mutant [23,34]. These results indicate that Q_6 itself may interact with certain Coq polypeptides and enhance formation of later Q-intermediates. Surprisingly, HHAB (a product of the Coq6 step) was detected in the *coq6* mutant cultured with exogenous Q_6 (Fig. 9B). It is possible that the presence of Q_6 facilitates the function of another hydroxylase; such a scenario has been reported for hydroxylases in *E. coli* Q_8 biosynthesis [86]. However, HHAB as identified in Fig. 9, may actually have the hydroxyl substituent located in another position on the ring. Determination of this will require purification of the intermediate and structural characterization.

In contrast, addition of exogenous Q_6 had no discernable effect on the Coq4, Coq7, or Coq9 indicator polypeptides or late-stage Q-intermediates in the *coq1*, *coq2*, *coq5* or *coq8* null mutants (Fig. 7 and Table 3). The steady-state levels of Coq1, Coq2, and Coq5 polypeptides are not affected by deletions in any of the other *COQ* genes [20]. While Coq8 over-expression in the *coq1* or *coq2* null mutants has little effect, Coq8 over-expression in the *coq5* null mutant allows production of $DDMQ_6$, and enhances steady-state levels of the Coq4, Coq6, Coq9 and Coq7 polypeptides [17]. Because Coq5 physically interacts with the other core-Coq polypeptides, yet the stabilizing effect of exogenous Q_6 on the other Coq polypeptides is not observed in the *coq5* null mutant, the Coq5 polypeptide is required for the interaction of Q_6 with CoQ-

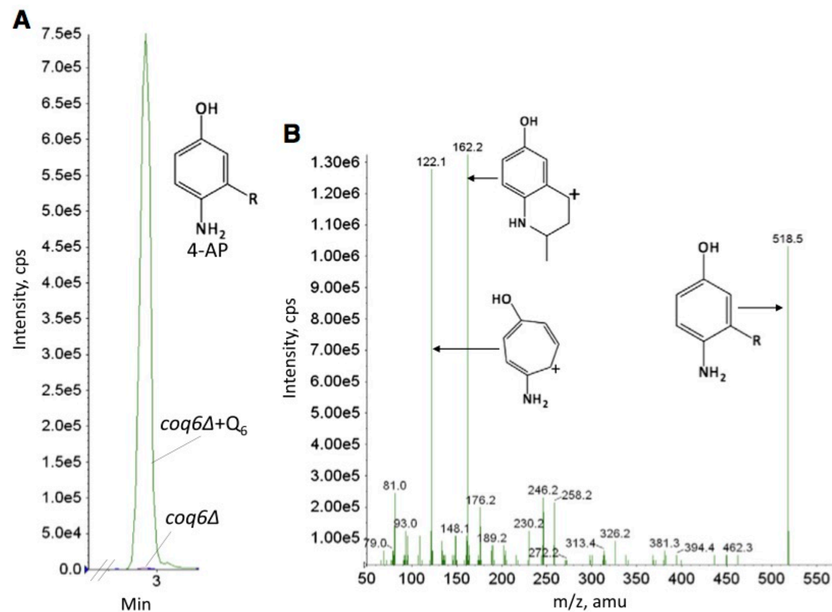

Fig. 10. Exogenous Q_6 increases the accumulation of 3-hexaprenyl-4-aminophenol (4-AP) in the *coq6* null mutant. Lipid extracts were prepared from the cell pellets of *coq6* null mutant yeast following growth in YPD with either the presence ($+Q_6$) or absence of Q_6 and analyzed by RP-HPLC-MS/MS as described in Fig. 9. MRM detected precursor-to-product ion transitions 518.4/122.0 (4-AP) and 455.4/197.0 (Q_4). In panel *A*, the green trace designates the 4-AP signal in the $+Q_6$ condition, and the purple trace designates the 4-AP signal in the absence of added Q_6 (*coq6Δ*). The peak areas of 4AP normalized by peak areas of Q_4 are 0.008 in *coq6Δ* and 2.68 in *coq6Δ* + Q_6. Panel *B* shows the fragmentation spectrum for the 4-AP $[M + H]^+$ precursor ion ($C_{36}H_{56}NO^+$; monoisotopic mass 518.4), the 4-AP tropylium ion $[M]^+$ ($C_7H_8NO^+$; 122.06), and the 4-AP chromenylium ion $[M]^+$ ($C_{10}H_{12}NO^+$; 162.1).

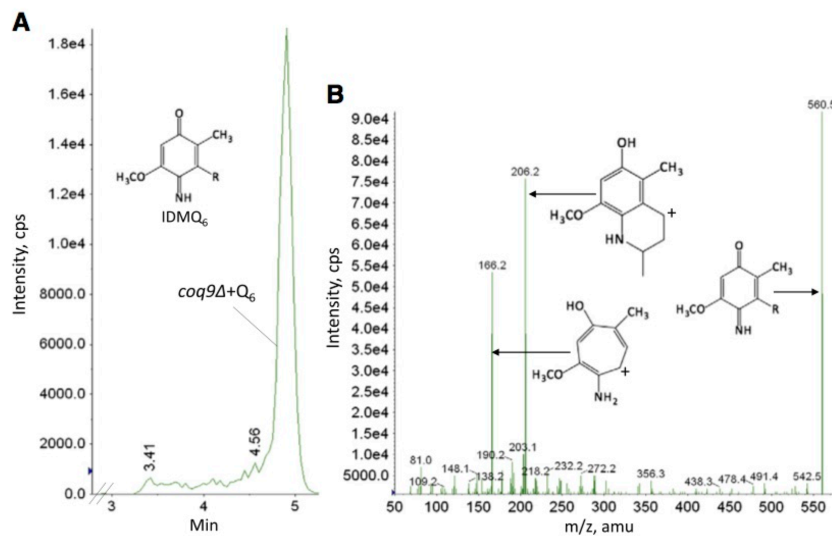

Fig. 11. Exogenous Q_6 leads to the accumulation of imino-demethoxy-Q_6 ($IDMQ_6$) in the *coq9* null mutant. Lipid extracts were prepared from cell pellets of the *coq9* null mutant yeast following growth in YPD with either the presence ($+Q_6$) or absence of Q_6 and analyzed by RP-HPLC-MS/MS as described in Fig. 8. Panel *A* shows the MRM detected precursor-to-product ion transition 560.5/166.2 ($IDMQ_6$). Panel *B*, shows the fragmentation spectrum for the $IDMQ_6$ $[M + H]^+$ precursor ion ($C_{38}H_{58}NO^+$; monoisotopic mass 560.4), the $IDMQ_6$ tropylium ion $[M]^+$ ($C_9H_{12}NO_2^+$; 166.1), and the $IDMQ_6$ chromenylium ion $[M]^+$ ($C_{12}H_{16}NO_2^+$; 206.1).

Table 3
Summary of the effect of over-expressing Coq8 or supplementation with exogenous Q_6.

Strains	No treatment					+hcCOQ8					+10 μM Q_6				
	Polypeptides			Intermediates from:		Polypeptides			Intermediates from:		Polypeptides			Intermediates from:	
	Coq4	Coq7	Coq9	4HB	pABA	Coq4	Coq7	Coq9	4HB	pABA	Coq4	Coq7	Coq9	4HB	pABA
WT	+	+	+	Q_6	Q_6	N/A	N/A	N/A	N/A	N/A	+	+	+	Q_6	Q_6
Coq1Δ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Coq2Δ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Coq3Δ	−	−	−	HHB	HAB	+	+	+	HHB	HAB	+	−	+	HHB	HAB
Coq4Δ	−	−	−	HHB	HAB	−	+	+	HHB	HHAB	−	−	+	HHB	HHAB
Coq5Δ	−	−	−	HHB	HAB	+	+	+	$DDMQ_6$	$DDMQ_6$	−	−	−	HHB	HAB
Coq6Δ	−	−	−	HHB	HAB	+	+	+	4-HP	4-AP	+	+	+	HHB	HHAB, 4-AP
Coq7Δ	−	−	−	HHB	HAB	+	−	+	DMQ_6	DMQ_6	+	−	+	DMQ_6	DMQ_6
Coq8Δ	−	−	−	HHB	HAB	+	+	+	Q_6	Q_6	−	−	−	HHB	HAB
Coq9Δ	−	−	−	HHB	HAB	+	+	−	DMQ_6	$IDMQ_6$	−	−	−	N/A	$IDMQ_6$

synthome (Fig. 12). On the other hand, there is no evidence that the Coq1 or Coq2 polypeptides are physically associated with the CoQ-synthome. In fact steady-state levels of indicator Coq polypeptides in the *coq1* null mutant are restored by expression of diverse polyisoprenyl-diphosphate synthases, including those from species that do not produce Q and hence would not be expected to interact with Coq polypeptides in yeast [36]. These findings support the interpretation that the stabilizing effects of exogenously added Q_6 or over-expression of Coq8 must depend on the synthesis of an endogenously produced polyisoprenyl-intermediate, such as HHB or HAB (Fig. 12).

In this study, steady-state levels of Coq1 are decreased upon supplementation of the *coq* null mutants with exogenous Q_6. It is tempting to speculate that Coq1 may play a regulatory step in the pathway where increases in Q_6 lead to a decrease in Coq1 polypeptide levels. This effect is not observed in wild type perhaps because supplementation with exogenous Q_6 does not have a significant impact on mitochondrial Q_6 content in wild-type yeast [70]. In contrast supplementation with exogenous Q_6 dramatically increases mitochondrial content of Q_6 in the *coq* null mutants, provided steps of endocytosis required for Q_6 uptake and trafficking to mitochondria are retained [70,84].

Dietary supplementation with Q_{10} can be an effective treatment for patients with partial defects in Q biosynthesis [87,88], and also shows benefit in mouse and *C. elegans* models of Q deficiency [89–92], and in cell culture models of mitochondrial diseases [93,94]. In this study we found that inclusion of exogenous Q_6 in the growth medium increased steady-state levels of mitochondrial polypeptides involved in respiratory electron transport and the citric acid cycle, including Rip1 (a subunit of complex III), Atp2 (a subunit of F_1 of complex V), and Mdh1, malate dehydrogenase. Previous studies indicated that supplemented Q_6 in *coq7* null mutant yeast restored steady-state levels of Atp2, cytochromes *c* and c_1, as well as porin, a mitochondrial outer membrane protein [95]. Exogenously supplied Q is converted to QH_2 by the respiratory chain, and QH_2 would exert its well known antioxidant effect on the mitochondrial membrane compartment and associated proteins [6]. Endogenous (hydroquinone) Q-intermediates formed after over-expression of Coq8 might also act as antioxidants. It is also possible that exogenous Q restored mitochondrial protein levels by increasing the content of mitochondria in *coq* null mutants. Q deficiencies may result from mitochondrial mutations affecting other processes; this is consistent with the observed effects of Q deficiency on mitophagy, and the inhibition of mitophagy by Q supplementation [93,94]. The findings presented here suggest that Q supplementation may correct defects in mitochondrial function through its beneficial effects in stabilizing the CoQ-synthome and de novo biosynthesis of Q, as well as contributing to enhanced respiratory electron transport and mitochondrial metabolism.

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Fig. 12. Proposed model for the yeast CoQ-synthome. This model is consistent with co-precipitation studies in *S. cerevisiae* with tagged Coq polypeptides, and the association of several of the Coq polypeptides in high molecular mass complexes, as assayed in digitonin extracts of mitochondria separated by two-dimensional blue native/SDS PAGE. The over-expression of Coq8, a putative kinase, is required to observe phosphorylated forms of Coq3, Coq5, and Coq7 [31]. Coq10, a START domain polypeptide, binds to Q and is postulated to act as a Q chaperone that delivers Q to the CoQ-synthome and/or the bc_1 complex [38]. Coq4 is denoted as a scaffolding protein, with binding sites for Q or polyisoprenyl-intermediates and serves to organize the high molecular mass Q biosynthetic complexes. See text for additional explanation.

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Chapter 3

Yeast Coq9 controls deamination of coenzyme Q intermediates that derive from *para*-aminobenzoic acid

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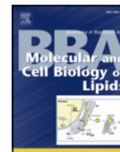

Yeast Coq9 controls deamination of coenzyme Q intermediates that derive from *para*-aminobenzoic acid☆

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ABSTRACT

Coq9 is a polypeptide subunit in a mitochondrial multi-subunit complex, termed the CoQ-synthome, required for biosynthesis of coenzyme Q (ubiquinone or Q). Deletion of *COQ9* results in dissociation of the CoQ-synthome, but over-expression of Coq8 putative kinase stabilizes the CoQ-synthome in the *coq9* null mutant and leads to the accumulation of two nitrogen-containing Q intermediates, imino-demethoxy-Q_6 ($IDMQ_6$) and 3-hexaprenyl-4-aminophenol (4-AP) when *para*-aminobenzoic acid (pABA) is provided as a ring precursor. To investigate whether Coq9 is responsible for deamination steps in Q biosynthesis, we utilized the yeast *coq5-5* point mutant. The yeast *coq5-5* point mutant is defective in the *C*-methyltransferase step of Q biosynthesis but retains normal steady-state levels of the Coq5 polypeptide. Here, we show that when high amounts of $^{13}C_6$-pABA are provided, the *coq5-5* mutant accumulates both $^{13}C_6$-imino-demethyl-demethoxy-Q_6 ($^{13}C_6$-$IDDMQ_6$) and $^{13}C_6$-demethyl-demethoxy-Q_6 ($^{13}C_6$-$DDMQ_6$). Deletion of *COQ9* in the yeast *coq5-5* mutant along with Coq8 over-expression and $^{13}C_6$- pABA labeling leads to the absence of $^{13}C_6$-$DDMQ_6$, and the nitrogen-containing intermediates $^{13}C_6$-4-AP and $^{13}C_6$-$IDDMQ_6$ persist. We describe a *coq9* temperature-sensitive mutant and show that at the non-permissive temperature, steady-state polypeptide levels of Coq9-ts19 increased, while Coq4, Coq5, Coq6, and Coq7 decreased. The *coq9-ts19* mutant had decreased Q_6 content and increased levels of nitrogen-containing intermediates. These findings identify Coq9 as a multi-functional protein that is required for the function of Coq6 and Coq7 hydroxylases, for removal of the nitrogen substituent from pABA-derived Q intermediates, and is an essential component of the CoQ synthome.

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1. Introduction

Coenzyme Q (ubiquinone or Q) is a polyprenylated benzoquinone lipid essential in cellular energy metabolism [1]. Q has a redox active benzoquinone ring connected to a polyisoprenoid side chain and is anchored to the mitochondrial inner membrane by the polyisoprenyl tail. The polyisoprenyl chain contains six units in *Saccharomyces cerevisiae* (Q_6), eight units in *Escherichia coli* (Q_8), and ten units in humans (Q_{10}) [2]. The reversible reduction and oxidation of the quinone/hydroquinone (Q/QH_2) enables its function as an electron and proton carrier in the mitochondrial respiratory chain and as a lipid-soluble antioxidant present in cellular membranes and in lipoproteins [1].

Q biosynthesis in *S. cerevisiae* requires nine Coq polypeptides (Coq1–Coq9), ferredoxin (Yah1), and ferredoxin reductase (Arh1) [3]. In addition, a Q-binding protein (Coq10) is required for efficient Q biosynthesis and for Q function as an electron carrier in respiratory electron transport [4]. 4-hydroxybenzoic acid (4HB) and *para*-aminobenzoic acid (pABA) both function as aromatic ring precursors for Q_6 biosynthesis in *S. cerevisiae* [3,5] (Fig. 1). Coq1 synthesizes the hexaprenyl diphosphate tail, which Coq2 attaches to ring precursors. Coq3 performs two *O*-methylation steps, Coq5 catalyzes *C*-methylation, and Coq6 and Coq7 catalyze hydroxylation steps. The proteins responsible for several steps in the Q biosynthesis pathway remain unknown, and the

Abbreviations: 4-AP, 3-hexaprenyl-4-aminophenol; $DDMQ_6$, demethyl-demethoxy-Q_6; DMQ_6, demethoxy-Q_6; HAB, 3-hexaprenyl-4-aminobenzoic acid; 4HB, 4-hydroxybenzoic acid; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; 4-HP, 3-hexaprenyl-4-hydroxyphenol; $IDDMQ_6$, imino-demethyl-demethoxy Q_6; $IDMQ_6$, imino-demethoxy-Q_6; mc*COQ8*, multi-copy *COQ8*; pABA, *para*-aminobenzoic acid; RP-HPLC-MS/MS, reverse phase-HPLC-MS/MS; Q, Coenzyme Q.

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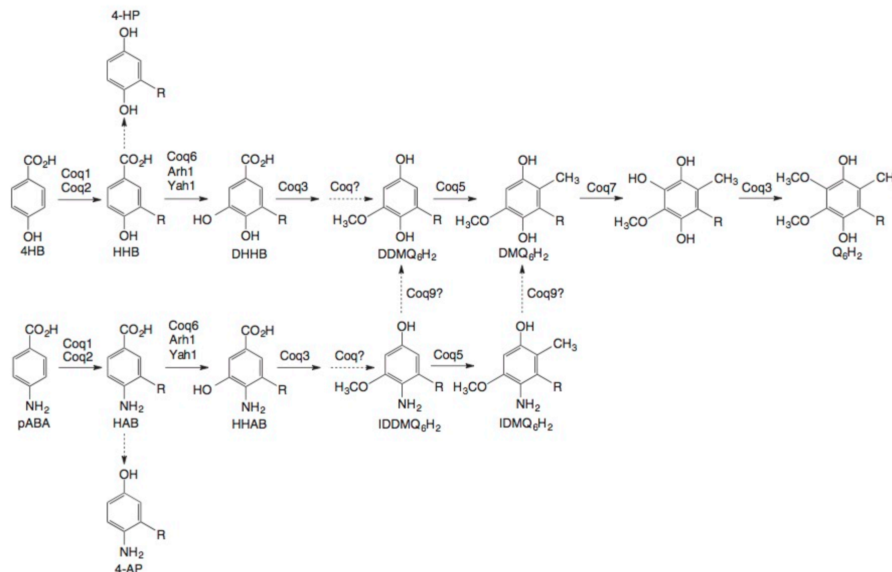

Fig. 1. Biosynthesis of Q in *S. cerevisiae* from 4HB or pABA. *S. cerevisiae* uses either 4-hydoxybenzoic acid (4HB) or *para*-aminobenzoic acid (pABA) as an aromatic ring precursor for Q_6 biosynthesis. Coq1 synthesizes the hexaprenyl-diphosphate tail and Coq2 attaches it to either ring. Coq6 performs the C5-hydoxylation reaction in concert with ferredoxin (Yah1) and ferredoxin reductase (Arh1). Coq3 catalyzes the two *O*-methylation steps and Coq5 catalyzes the C-methylation step. Coq9 is the putative deaminase that removes the amino groups on imino-demethyl-demethoxy-Q_6 (IDDMQ_6) or imino-demethoxy-Q_6 (IDMQ_6). Coq9 is also required for Coq7 to catalyze the penultimate hydroxylation step, and for efficient C5-hydroxylation by Coq6.

functional roles of the Coq4, Coq8, and Coq9 polypeptides still need further characterization (Fig. 1).

Coq9 is a polypeptide subunit in the Q biosynthetic complex. Similar to the other Coq polypeptides (with the exception of Coq2, an integral membrane protein), Coq9 is peripherally associated to the inner mitochondrial membrane facing the matrix side [6,7]. Coq9 co-migrates with Coq3 and Coq4 at high molecular mass and HA tagged Coq9 co-purifies with Coq4, Coq5, Coq6, and Coq7 [6,7]. Recovery of tagged versions of Coq3, Coq6, or Coq9 from digitonin extracts of yeast mitochondria results in the recovery of the CoQ-synthome, a multi-subunit Q-biosynthetic complex, containing Coq3–Coq9 polypeptides, Q_6, Q_6 intermediates, as well as other partner proteins, including the newly identified Coq11 [8]. Deletion of any one of the *COQ3–COQ9* genes leads to the decreased steady state of several of the other Coq polypeptides and to the accumulation of two early Q intermediates, 3-hexaprenyl-4-hydroxybenzoic acid (HHB) and 3-hexaprenyl-4-aminobenzoic acid (HAB) [6,9]. Sensitive Coq polypeptides were stabilized and late-stage Q intermediates accumulated in some of the *coq3-coq9* null mutants that over-expressed Coq8, a putative kinase [10]. Conserved kinase motifs in Coq8 are essential for the phosphorylation of Coq3, Coq5, and Coq7 [11,12], and Coq8 over-expression stabilized the Q biosynthetic complex in yeast [7]. These studies suggest that Coq8 over-expression might stabilize the complex by phosphorylation. Recent work identified auto-phosphorylation and ATPase activity in ADCK3, a human ortholog of yeast Coq8 [13,14].

Several studies suggest that yeast Coq9 is important for formation or stability of the CoQ synthome [7]. Coq8 over-expression suppressed the Q-less phenotype of the *coq9* point mutant yeast strain C92 [15]. C92 has a nonsense point mutation in the *coq9* gene causing an early stop codon; Coq8 over-expression increased the steady-state level of the Coq9 polypeptide in the C92 mutant [6]. Other work utilizing Coq8 over-expression showed that yeast Coq9 is important for correct function of Coq7 [10]. When Coq8 is over-expressed, intermediates that accumulate in the yeast *coq9* null mutant were also found to accumulate in the *coq7* null mutant. For example, with Coq8 over-expression, $^{13}C_6$-DMQ_6 accumulates in both yeast *coq9* and *coq7* null mutants when $^{13}C_6$-4HB was provided as an aromatic ring precursor [10,16]. However, when the same strains were provided with $^{13}C_6$-pABA, the yeast *coq9* null mutant with Coq8 over-expression accumulated $^{13}C_6$-imino-demethoxy Q_6 ($^{13}C_6$-IDMQ_6), while under the same labeling conditions, the yeast *coq7* null mutant with Coq8 over-expression still produced $^{13}C_6$-DMQ_6 [10]. This finding suggests that Coq9 is required for Coq7 function but is also required for deamination of Q intermediates when pABA is used as a ring precursor. While pABA is utilized to generate Q_6 in yeast, it is not a ring precursor for Q biosynthesis in human, mouse, *Arabidopsis thaliana*, or *E. coli* [17,18]. Therefore, the important role that Coq9 plays in the deamination of Q intermediates might be unique to yeast Coq9. Coq9 is also necessary for correct function of Coq6, because in the presence of Coq8 over-expression, both *coq6* null and *coq9* null mutants accumulate $^{13}C_6$-4-HP (upon labeling with $^{13}C_6$-4HB) and $^{13}C_6$-4-AP (upon labeling with $^{13}C_6$-pABA) [10]. In this study, we examined the role of yeast Coq9 in mediating the deamination of other nitrogen-containing Q intermediates and employed a temperature-sensitive mutant to further clarify its role in stabilizing the CoQ synthome.

2. Materials and methods

2.1. Yeast strains and growth media

S. cerevisiae strains used in this study are listed in Table 1. Growth media used in this study were prepared as described [19] and included YPD (2% glucose, 1% yeast extract, 2% peptone), YPEG (1% yeast extract, 2% peptone, 2% ethanol, and 3% glycerol), YPGal (1% yeast extract, 2% peptone, 2% galactose, 0.1% dextrose), and YPG (3% glycerol, 1% yeast extract, 2% peptone). Synthetic Dextrose/Minimal medium (SD-

Table 1
Genotype and Source of Yeast Strains.

Strain	Genotype	Source
W3031B	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303Δcoq4	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq4::TRP1*	[41]
W303Δcoq5	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq5::HIS3*	[20]
W303Δcoq6	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq6::LEU2*	[47]
W303Δcoq7	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq7::LEU2*	[42]
W303Δcoq9	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq9::URA3*	[15]
BY4741Δcoq9	MAT a *coq9Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	[43][b]
CH316-6B	MAT α *coq5-5 trp1-1 ura3-1*	[27]
CH316-6BΔcoq9	MAT α *coq5-5 trp1-1 ura3-1 coq9::kanMX4*	This study

[a] Dr. Rodney Rothstein, Department of Human Genetics, Columbia University.
[b] European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF), available on-line.

complete) consisted of 0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, and amino acids were added to final concentrations as described [20]. Selective SD/Minimal medium lacking uracil or leucine (SD–Ura or SD–Leu) were similarly prepared. Agar plate media were prepared as described above and included 2% bacto agar (Fisher).

2.2. Disruption of COQ9 in the CH316-6B (coq5-5 point mutant) yeast strain

A PCR product containing the KanMX4 gene was amplified with the genomic DNA isolated from BY4741Δ*coq9* (used as template) and with primers that annealed to 100 bp upstream and downstream of the *COQ9* ORF. The sequences of the primers utilized were 5′-TTTGGGCCTA CATAAGGTACTTC-3′ and 5′-CGCACAGTACCAATAAATCTGCC-3′. The PCR product was then transformed into the yeast *coq5-5* point mutant strain (CH316-6B) to create CH316-6BΔcoq9. Transformants that grew on YPD + 200 μg/ml G418 (Geneticin) were selected. Proteins were extracted from these transformants as described [21] and separated by SDS–PAGE with a 10% polyacrylamide gel. Proteins were transferred to an Immobilon-P transfer membrane (Millipore) and analyzed by immunoblotting as described [7]. The primary antibody against Coq9 was used at a 1:1000 dilution and the secondary antibody, goat anti-rabbit IgG H&L chain-specific peroxidase conjugate (Calbiochem), at a 1:10,000 dilution. The absence of Coq9 polypeptide confirmed that *COQ9* was replaced with KanMX4.

2.3. Construction of plasmids

Plasmids used in this study are listed in Table 2. Over-expression of Coq8 made use of the p4HN4 plasmid (mc*COQ8*), which contains the *COQ8* gene in pRS426, a multi-copy yeast shuttle vector [22]. To construct the plasmid pRS315COQ9 (*COQ9*), the genomic DNA of W3031B was isolated using the Wizard Genomic DNA purification kit (Promega). The *COQ9* gene was then amplified with Taq polymerase and primers XhoI400upCoq9F (5′-CTCGAGCCGGGTTCAGAGGTAAAAGG-3′ −400 to −380 of *COQ9* with XhoI restriction site at the 5′ end) and BamHI240downCoq9R (5′-GGATCCGGGACAAGCAGGAAGAACTA-3′ +220 to +240 with BamHI restriction site at the 5′ end). PCR products were inserted into the TOPO vector using the TOPO TA Cloning kit (Invitrogen) resulting in a plasmid named TOPOCOQ9. pRS315 and TOPOCOQ9 were digested with the restriction digestion enzymes XhoI and BamHI (New England BioLabs) and separated by gel electrophoresis. DNA fragments that contained the digested pRS315 or *COQ9* were purified from agarose gel using the Purelink quick DNA gel extraction kit (Invitrogen) and then ligated with T4 DNA Ligase (New England BioLabs) resulting in pRS315COQ9. The correct nucleotide sequence of the *COQ9* ORF in pRS315COQ9 was verified (Laragen, Los Angeles). pRS315COQ9 was shown to rescue growth of W303ΔCOQ9 on medium containing a non-fermentable carbon source, YPG.

Table 2
Plasmid constructs used in this study.

Plasmid	Relevant genes	Copy number	Source
pRS315	Yeast shuttle vector	Low copy	[44]
pRS426	Yeast shuttle vector	Multi-copy	[43,44]
p4HN4 (mcCOQ8)	Yeast *ABC1/COQ8*	Multi-copy	[45]
pRS315COQ9 (*COQ9*)	Yeast *COQ9*	Low copy	This work
TS19	Yeast *COQ9*	Low copy	This work
E55G	Yeast *COQ9*	Low copy	This work
R107G	Yeast *COQ9*	Low copy	This work
Q256L	Yeast *COQ9*	Low copy	This work
a-12g	Yeast *COQ9*	Low copy	This work
a-93g	Yeast *COQ9*	Low copy	This work
E55GR107G	Yeast *COQ9*	Low copy	This work
E55GQ256L	Yeast *COQ9*	Low copy	This work
R107GQ256L	Yeast *COQ9*	Low copy	This work
R107GQ256L	Yeast *COQ9*	Low copy	This work
E55GR107GQ256L	Yeast *COQ9*	Low copy	This work
R107GQ256La-93g	Yeast *COQ9*	Low copy	This work
R107GQ256La-12g	Yeast *COQ9*	Low copy	This work
E55GR107Ga-12g	Yeast *COQ9*	Low copy	This work

2.4. Construction of coq9 temperature-sensitive mutants using polymerase chain reaction (PCR) mutagenesis

Temperature-sensitive *coq9* yeast strains were generated using error-prone PCR, followed by *in vivo* homologous recombination [23]. *COQ9* with 400 bp 5′- and 240 bp 3′-flanking regions was cloned into pRS315, resulting in the plasmid pRS315COQ9. *COQ9* was amplified using PCR with primers designed 180 bp upstream (5′-ACTGGAAAGC GGGCAGTGA-3') and 240 downstream (5′-CAAGTGTAGCGGTCACGC TG-3′) of the multiple cloning region in the presence of 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP, 0.05 mM dATP, 0.8 mM $MgCl_2$, and 0.6–0.8 mM $MnCl_2$. The amplified fragments were purified and co-transformed with linearized pRS315 into the yeast null mutant W303Δ*coq9*. Leu^+ transformants were then selected and screened for growth at 25 °C and 37 °C on YPEG plates. One of the plasmids generated (TS19) using the method outlined here was used in this study.

2.5. Site-directed mutagenesis of S. cerevisiae COQ9

Mutagenesis of the wild-type yeast *COQ9* was carried out with either the QuikChange or QuikChange Lightning site-directed mutagenesis kit from Agilent following the manufacturer's protocol. The primers used to generate single mutant plasmids are listed in Table 4: for E55G mutant, forward primer E55Gf and reverse primer E55Gr; for R107G mutant, forward primer R107Gf and reverse primer R107Gr; for Q256L mutant, forward primer Q256Lf and reverse primer Q256Lr; for a-12g mutant, forward primer a-12gf and reverse primer a-12gr; for a-93g mutant, forward primer a-93gf and reverse primer a-93gr. Single mutant plasmids were transformed into *E. coli* as described in the manufacturer's

Table 3
Description and source of antibodies.

Antibody	Working dilution	Source
Atp2	1:4000	Carla M. Koehler[a]
Coq4	1:250	[46]
Coq5	1:5000	[27]
Coq6	1:250	[47]
Coq7	1:1000	[48]
Coq9	1:1000	[6]

[a] Dr. Carla M. Koehler, Department of Chemistry and Biochemistry, UCLA

Table 4
Primer sequences (site-directed mutagenesis of *S. cerevisiae COQ9*).

Primer name	Sequence
E55Gf	5′-AGAGAAACCGTGCCCG GGAACAAAC-3′
E55Gr	5′-GTTTGTTCCCGGGCACGGTTTCTCT-3′
R107Gf	5′-GGGTTGATTCCTTCAGTTAAACGATACCCTTTATCTACC-3′
R107Gr	5′-GGTAGATAAAGGGTATCGTTTAACTGAAGGAATCAACCC-3′
Q256Lf	5′-CCCCTAACTAATAGAGATTTGATTAAATTTA CCGTAGACA-3′
Q256Lr	5′-TGTCTACGGTAAATTTAATCAAATCTC TATTAGTTAGGGG-3′
a-12gf	5′-GAGATAACAGAGTCTTTACCGCATTATAAATC-3′
a-12gr	5′-GATTTATAATGCGGTAAAGACTCTGTTATCTC-3′
a-93gf	5′-GCAATAACAATAGTAAGAAACGATAATACGGGG-3′
a-93gr	5′-CCCCGTATTATCGTTTCTTACTATTGTTATTGC-3′

protocol (Agilent) and then were purified from 3 ml cultures. The identities of the mutations were verified by DNA sequencing (Laragen). To generate secondary point mutations, single mutant plasmids were used as templates: E55GR107G mutant was generated using E55G as template and the R107Gf and R107Gr as primers; E55GQ256L mutant was generated using E55G as template and the Q256Lf and Q256Lr as primers; R107GQ256L mutant was generated using R107G as template and the Q256Lf and Q256Lr as primers. To generate tertiary point mutations, double mutant plasmids were used as templates: E55GR107GQ256L mutant was generated using R107GQ256L as template and the E55Gf and E55Gr as primers; R107GQ256La-12g mutant was generated using R107GQ256L as template and the a-12gf and a-12gr as primers; R107GQ256La-93g mutant was generated using R107GQ256L as template and the a-93gf and a-93gr as primers; E55GR107Ga-12g mutant was generated using E55GR107G as template and the a-12gf and a-12gr as primers. The identities of these mutations were verified by DNA sequencing (Laragen).

2.6. Lipid extraction and detection of Q_6 intermediates by HPLC and tandem mass spectrometry

The designated strains of *coq5-5* yeast mutants were labeled with $^{13}C_6$-pABA followed by lipid analysis. Labeling media were prepared with 50 μg/ml $^{13}C_6$-pABA dissolved in ethanol. The final concentration of ethanol in the medium was 0.2%. Yeast mutants without plasmids or harboring p4HN4 (mcCOQ8) were grown in 100 ml of SD complete or SD–Ura, respectively. To label cells, yeast cultures were diluted to 0.5 A_{600nm}/ml in 100 ml of fresh SD complete or SD–Ura with $^{13}C_6$ pABA and labeled for 6 hours. The final cell density was 2–3 A_{600nm}/ml. For lipid extraction, cells were collected by centrifugation and 145 pmol Q_4 was added to each cell pellet to serve as an internal standard. Lipid extracts were analyzed by RP-HPLC-MS/MS [10]. Briefly, a phenyl-hexyl column (Luna 5u, 100 × 4.60 mm, 5 μm, Phenomenex) was used for liquid chromatography. The mobile phase includes Solvent A (methanol/isopropanol, 95:5, with 2.5 mM ammonium formate) and Solvent B (isopropanol, 2.5 mM ammonium formate). From 0 to 6 min, Solvent B was increased linearly from 0 to 5%, and the flow rate was increased from 600 to 800 μl/min. At 7 min, the flow rate and mobile phase were changed back to 100% Solvent A and a flow rate of 600 μl/min. The 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA) was used for multiple reaction monitoring mode (MRM) analysis. Data were processed with Analyst version 1.4.2 software (Applied Biosystems).

To quantify Q_6 content and determine *de novo* synthesis of Q_6 intermediates in temperature-sensitive yeast mutants at permissive (25 °C) and non-permissive (37 °C) temperatures, yeast cells were labeled with $^{13}C_6$-pABA followed by lipid analysis as described above. Labeling media were prepared with 10 μg/ml $^{13}C_6$-pABA dissolved in ethanol (ethanol was 0.2% final concentration). Cells were collected (a total of 30 A_{600nm}) as pellets after 5 hours of labeling. Q_4 was added (145 pmol) to each cell pellet as an internal standard. The exact amounts (total pmol) of ^{12}C-Q_6 and $^{13}C_6$-Q_6 were calculated by normalizing the peak areas of $^{12}C_6$-Q_6 (sum of oxidized and reduced) and $^{13}C_6$-Q_6 (sum of oxidized and reduced) by the peak areas of Q_4 (sum of oxidized and reduced); the pmol amounts were then determined from the Q_6 standard curve. After the pmol of ^{12}C-Q_6 and $^{13}C_6$- Q_6 was calculated, they were further normalized by the wet weight of yeast pellets. Chemical standards for Q intermediates 4-AP, IDMQ_6, and DMQ_6 are not available. To quantify these intermediates, the peak areas for each were normalized by the recovery of Q_4 (sum of oxidized and reduced peaks). Finally, calculated values were further normalized by the wet weight of yeast pellets.

2.7. Mitochondrial isolation and immunoblot analyses with temperature-sensitive mutants

To study the protein levels in temperature-sensitive mutants, mitochondria were isolated from yeast cells and analyzed by immunoblot. Yeast cultures were grown to 3–4 A_{600nm} in YPGal medium at different temperatures (W3031B and W303Δ*coq9*:TS19 were grown at 25 °C and 37 °C for 18.5 hours; BY4741Δcoq9, W303Δcoq7, and W303Δcoq4, W303Δcoq5, and W303Δcoq6 were grown at 30 °C overnight). Crude mitochondria were isolated from a total volume of 1 L of culture as described [24]. Next, crude mitochondria were further purified with an OptiPrep discontinuous iodixanol gradient as described in [7]. Purified mitochondria (15 μg based on total protein measured by the bicinchoninic acid assay from Thermo) were separated by SDS–PAGE with 10% polyacrylamide gels. Proteins were transferred to Immobilon-P transfer membranes (Millipore) and analyzed by immunoblotting as described [7]. The source and use of primary antibodies is described in Table 3. The secondary antibody used was goat anti-rabbit IgG H&L chain specific peroxidase conjugate (Calbiochem), 1:10,000.

2.8. RNA extraction and Northern blot analyses with temperature-sensitive mutants

To determine the *COQ* mRNA levels in temperature-sensitive mutants, samples of yeast total RNA were analyzed by Northern blot. Yeast cells were grown to 0.5 A_{600nm} in YPGal; W3031B and W303Δ*coq9*:TS19 were grown at 25 °C and 37 °C for 18.5 hours. Aliquots (25 ml) of each culture were harvested by centrifugation at 1000 × g for 5 min at 4 °C, and cell pellets were washed with water and frozen in liquid nitrogen. RNA was extracted as described in [25] with some modifications. Briefly, 500 μl of RNA–SDS buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM EDTA, pH 8.0, 2% SDS), 400 μl of acid-washed glass beads (Sigma), and 500 μl RNA Phenol-Chloroform (Fisher) were added. The tubes were vortexed for 1 min and incubated for 6 min at 65 °C. An aliquot (450 μl) of the aqueous top layer was added to 450 μl fresh RNA Phenol-Chloroform for the second extraction. RNA was precipitated with 1 ml ethanol and 40 μl 3 M sodium acetate (pH 5.2). The RNA pellets were then washed with 450 μl 70% ethanol (v/v) and resuspended in distilled water.

Samples of RNA (5 μg) were denatured at 55 °C for 1 hour with 5 volumes of glyoxal buffer. (Glyoxal buffer contains 60.9% DMSO (v/v) (Sigma), 20.3% deionized glyoxal (v/v) (Fluka), 4.87% glycerol (v/v), 0.04 mg/ml ethidium bromide, and 12.2% 10× BPTE (v/v)). 10× BPTE contains 100 mM PIPES (Sigma), 300 mM BIS–TRIS (Sigma), and 10 mM EDTA. Samples of denatured RNA were separated by 1.2% agarose–1× BPTE gels and transferred to Hybond N^+ nylon membranes (GE Healthcare) in 10× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) as described [26]. Blots were crossed-linked and hybridized with Church hybridization buffer containing probes as described [25].

Probes were generated as described [25], with some modifications. Briefly, PCR products were generated with genomic DNA extracted from W3031B and the primers annealed to the ORF of *COQ4*, *COQ5*,

COQ6, *COQ7*, or *COQ9* as listed in Table 5. Next, 4 μl of PCR products (100–200 ng/μl) were used as a template and mixed with 2.1 μl of distilled H_2O, 4 μl of the mixture of rATP, rCTP, and rGTP (2.5 mM each), 2.4 μl of 100 μM rUTP, 2 μl of 100 mM DTT, 1.25 μl of [α-^{32}P] UTP (6000 Ci/mmol; 40 μCi/μl; Perkin Elmer), 4 μl of 5× Promega transcription buffer, and 0.25 μl of T3 RNA polymerase (Promega). The *in vitro* transcription reaction was incubated at 37 °C for 1 hour. To generate the *SCR1* probe, 0.2 μl of 5′-GTCTAGCCGCGAGGAAGG-3′ oligo (100 μM) was mixed with 1 μl of T4 polynucleotide kinase (PNK), 1 μl of 10× PNK buffer, 3 μl of [γ-^{32}P] ATP (3000 Ci/mmol; 10 μCi/μl; Perkin Elmer), and 4.8 μl of distilled H_2O. The reaction was incubated at 37 °C for 30 min.

3. Results

3.1. The deletion of COQ9 in coq5-5 point mutant yeast leads to the accumulation of unique nitrogen-containing Q intermediates

In addition to Coq6 and Coq7 function, Coq9 also appears to be necessary to convert $IDMQ_6$ to DMQ_6 (Fig. 1). To investigate whether Coq9 may act to remove the amino/imino group from other Q intermediates, we utilized the CH316-6B yeast strain that harbors the *coq5-5* point mutation. This mutant lacks *C*-methyltransferase activity but retains steady-state levels of Coq5 and other Coq polypeptides [27] and accumulates $DDMQ_6$ as a late-stage Q intermediate [28]. To examine the effect of *COQ9* on the $DDMQ_6$ intermediate, we deleted the *COQ9* gene in CH316-6B yeast to generate a double mutant strain (*coq5-5 Δcoq9*), and over-expressed Coq8 to stabilize the CoQ-synthome. We labeled the *coq5-5*, *coq5-5:mcCOQ8*, and *coq5-5 Δcoq9:mcCOQ8* yeast strains with $^{13}C_6$-pABA for 6 hours and used HPLC with tandem mass spectrometry to detect Q_6 intermediates in the yeast lipid extracts. We found that both *coq5-5* and *coq5-5:mcCOQ8* strains accumulated predominant amounts of $^{13}C_6$-$DDMQ_6$ as well as readily detectable levels of $^{13}C_6$-$IDDMQ_6$ (blue and green traces in Fig. 2A and B). However, in the double mutant *coq5-5 Δcoq9:mcCOQ8*, $^{13}C_6$-$DDMQ_6$ disappeared (Fig. 2B), while intermediates containing the nitrogen group, $^{13}C_6$-$IDDMQ_6$ and $^{13}C_6$-4-AP, accumulated (red traces in Fig. 2A and C). $^{13}C_6$-$DDMQ_6$ was identified by its retention time (4.59 min), precursor-to-product ion transition (553.4/159.0), and fragmentation spectrum [28]. $^{13}C_6$-$IDDMQ_6$ was identified by its retention time (4.41 min), precursor-to-product ion transition (552.4/158.0), and fragmentation spectrum (Fig. 3). $^{13}C_6$-4-AP was identified by its retention time (2.94 min), precursor-to-product ion transition (524.4/128.0), and fragmentation spectrum [7]. The results suggest that Coq9 is essential for converting the amino or imino group to a hydroxyl group in Q intermediates derived from pABA.

3.2. Characterization of a coq9 temperature-sensitive mutant (coq9-ts19)

To better understand the role of Coq9 in Q biosynthesis, we generated conditional *coq9* mutants. Using error-prone PCR and *in vivo* recombination, we mutagenized the cloned *COQ9* gene. One clone, TS19 was selected for further analysis (Fig. 4). At the permissive temperature of 25 °C, the yeast *coq9* null mutant harboring TS19 grew as well as wild type, while at the non-permissive temperature of 37 °C, it grew poorly when compared to either the wild type at 37 °C, or to growth at the permissive temperature, 25 °C.

Table 5
Primer sequences (Riboprobe generation).

Primer name	Sequence
COQ4F	5′-ACAGCTACTTTGCCAGTGAAATGCC-3′
COQ4T3R	5′-AATTAACCCTCACTAAAGGGAAGTCGTGGCTCGTTTCTGTGAGTTGT-3′
COQ5F	5′-TGTTGATTTCTTCACGGATCGTTCG-3′
COQ5T3R	5′-AATTAACCCTCACTAAAGGGAGCCAGCAGATTTGAATCCTGCCTTC-3′
COQ6F	5′-CAGGATTGTCAGTGTTACGCCTAGATC-3′
COQ6T3R	5′-AATTAACCCTCACTAAAGGGAGGGCAACTCTATCAGTGCAATAACGA-3′
COQ7F	5′-GCAGAGGCTTTTCCGTCTTATCATCT-3′
COQ7T3R	5′-AATTAACCCTCACTAAAGGGAGCCATATACGAATCATGCTTGATAGCGG-3′
COQ9F	5′-ATGCTTTGTCGCAATACTGCCAGAACG-3′
COQ9T3R	5′-AATTAACCCTCACTAAAGGGATACTCACCCAAACGCATGACCCTA-3′

The TS19 clone was sequenced to determine the mutations that caused the temperature-sensitive (ts) phenotype. Five mutations were detected in TS19: Adenine−12→Guanine (a−12g), Adenine−93 →Guanine (a−93g), Glu55→Gly (E55G), Arg107→Gly (R107G), and Gln256→Leu (Q256L). The first two mutations are upstream of the *COQ9* ORF, and the remaining three are within the *COQ9* ORF. To identify the amino acids that were critical for Coq9 function, *coq9* alleles were generated that contained single, double, or triple mutations. The plasmids generated were then transformed into the yeast *Δcoq9* null mutant (W303Δcoq9) and subjected to plate dilution assay. Serial dilutions were plated on SD−Leu to confirm the presence of the plasmid. WT was not transformed with any plasmid, so it had no growth on SD−Leu, but it grew well on YPG at both permissive and non-permissive temperatures. W303Δcoq9 harboring empty vector (EV) showed no growth on YPG at either temperature as expected. W303Δcoq9 harboring wild-type *COQ9* (COQ9) was included to provide a positive control for growth on YPG at the different temperatures. Yeast *Δcoq9* mutants harboring plasmids containing single mutations, E55G and Q256L, did not show altered growth at the non-permissive temperature, but the presence of the R107G single mutation did produce slightly defective growth at 37 °C (Fig. 4A). Yeast *Δcoq9* mutants harboring plasmids containing double mutations in combination with R107G had defective growth at 37 °C, but not with E55G Q256L (Fig. 4B). The combination of three amino acid substitution mutations E55G R107G Q256L recapitulated the TS19 phenotype (Fig. 4C). The presence of two mutations upstream of *COQ9* start codon had no effect on yeast growth at non-permissive temperature (Fig. 4). Therefore, the full temperature-sensitive phenotype of *Δcoq9*:TS19 requires the presence of E55G, R107G, and Q256L mutations.

3.3. Temperature-sensitive mutations in COQ9 lead to the destabilization of other Coq polypeptides at non-permissive temperature

Deletion of the yeast *COQ9* gene leads to the decreased steady state of other Coq polypeptides, especially Coq4 and Coq7 [6]. To determine whether the Coq9-ts19 polypeptide impacts other Coq polypeptide levels, we grew wild type (WT) and *Δcoq9*:TS19 (Δ9:TS19) yeast in YPGal for 18.5 hours at 25 °C or 37 °C, and then isolated mitochondria. The steady-state levels of Coq9, Coq4, Coq7, Coq5, and Coq6 in isolated mitochondria were analyzed by immunoblotting (Fig. 5). In wild-type mitochondria, Coq9, Coq4, and Coq7 levels were increased at 37 °C (Fig. 5A, B, and C). Expression of certain genes in *S. cerevisiae* can be induced or repressed in response to environmental changes, such as heat shock [29]. Therefore, the increased steady-state levels of Coq polypeptides at the non-permissive temperature might be a stress response. Coq5 and Coq6 levels were not changed at higher temperature (Fig. 5D and E). In the *Δcoq9*:TS19 mutant, steady-state polypeptide levels of Coq9-ts19 were increased at the non-permissive temperature (Fig. 5A), while Coq4, Coq7, Coq5, and Coq6 tended to be decreased at 37 °C (Fig. 5B, C, D, and E). The results suggest that at high temperature, the expression of Coq9-ts19 causes destabilization of the Coq polypeptide complex.

3.4. Changes in COQ RNA levels do not correspond to the observed changes in Coq polypeptide levels

To investigate whether the change of Coq polypeptides at different temperatures is a response at gene expression level or protein level, we analyzed the mRNA levels of *COQ4*, *COQ5*, *COQ6*, *COQ7*, and *COQ9* in WT and Δ9:TS19 yeast grown at either the permissive (25 °C) or

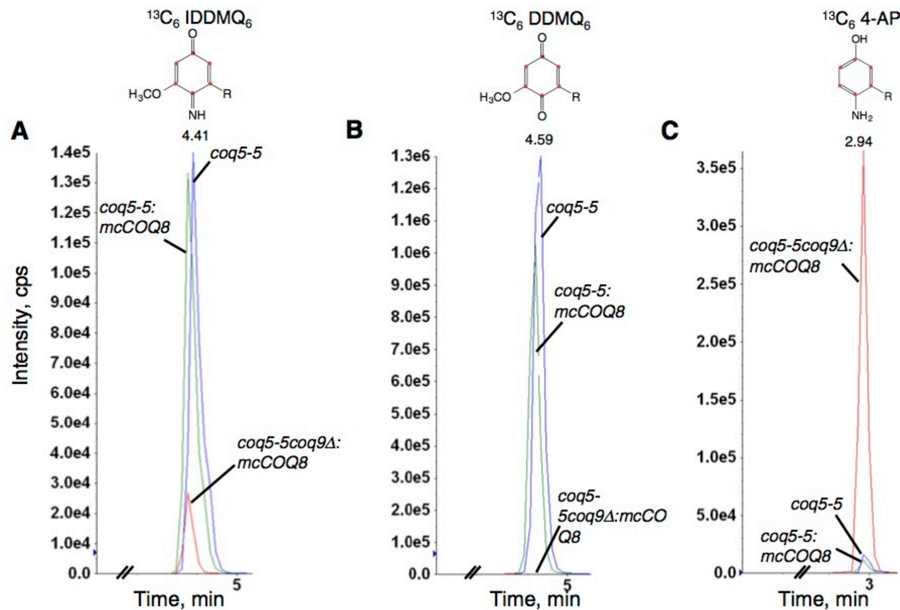

Fig. 2. The deletion of *COQ9* in a yeast *coq5-5* point mutant leads to the accumulation of 3-hexaprenyl-4-aminophenol (4-AP) and the disappearance of demethyl-demethoxy-Q_6 (DDMQ$_6$), but imino-demethyl-demethoxy-Q_6 (IDDMQ$_6$) is still present. Yeast *coq5-5* point mutants with *COQ8* over-expressed (*coq5-5:mcCOQ8*), without *COQ8* over-expressed (*coq5-5*), or with the *COQ9* gene deleted and *COQ8* over-expressed (*coq5-5 coq9Δ:mcCOQ8*), were cultured in SD complete or SD−Ura with 50 μg/ml $^{13}C_6$-pABA and 2 μl ethanol/ml medium at 0.5 A_{600nm}/ml and collected after 6 hours. Q_4 (145.4 pmol) was added prior to extraction to serve as an internal standard. Lipid extracts prepared from the cell pellets were analyzed by RP-HPLC-MS/MS. Multiple reaction monitoring (MRM) detected precursor-to-product ion transitions 552.4/158.0 ($^{13}C_6$ -IDDMQ$_6$), 553.4/159.0 ($^{13}C_6$ -DDMQ$_6$), and 524.4/128.0 ($^{13}C_6$-4-AP). Just the oxidized forms of IDDMQ$_6$ and DDMQ$_6$ were detected, while only the reduced form of 4-AP was present. $^{13}C_6$-DDMQ$_6$ accumulates in the *coq5-5* and *coq5-5:mcCOQ8* yeast mutants (*B*), and $^{13}C_6$-IDDMQ$_6$ is readily detected (*A*). $^{13}C_6$-IDDMQ$_6$ and $^{13}C_6$-4-AP accumulate in *coq5-5 coq9Δ:mcCOQ8* (*A* and *C*), but $^{13}C_6$-DDMQ$_6$ is not detected (*B*). In all panels, the *blue traces* designate the Q intermediate signals in *coq5-5* and *green traces* designate the Q intermediate signals in *coq5-5:mcCOQ8*, and the *red traces* indicate the Q intermediate signals in *coq5-5 coq9Δ:mcCOQ8*.

non-permissive temperatures (37 °C). The mRNA levels of *COQ4*, *COQ5*, *COQ6*, *COQ7*, and *COQ9* were not changed in the Δ9:TS19 mutant at different temperatures (Fig. 6). This was also the case for the mRNA levels of *COQ5*, *COQ7*, and *COQ9* in wild type (Fig. 6A, C, and D). The mRNA levels of *COQ4* and *COQ6* in wild-type yeast were decreased at non-permissive temperature (Fig. 6B and E). Thus, the increase observed in the steady-state polypeptide levels of Coq9, Coq4, Coq7, and Coq6 in wild-type mitochondria at 37 °C (Fig. 5A, B, and C) cannot be attributed to corresponding changes in mRNA content. Therefore, it seems most likely that changes in steady-state Coq polypeptide levels observed in Fig. 5 are instead due to the stabilization or destabilization of the CoQ-synthome.

3.5. Incubation at the non-permissive temperature leads to decreased Q_6 and the accumulation of nitrogen-containing intermediates in the yeast Δcoq9 mutant harboring TS19

The results in Figs. 4 and 5 suggest that the Coq9 polypeptide harboring the TS19 mutations (Coq9-ts19) is functionally impaired at the non-permissive temperature, 37 °C. To gain further insight into the nature of the Coq9-ts19 temperature-sensitive defects, we analyzed the *de novo* synthesis of Q_6 and Q_6 intermediates in the *Δcoq9*:TS19 mutant. Wild type (WT) and *coq9* null mutant harboring empty vector (*Δcoq9: EV*) were included as controls. Yeast were grown in selective liquid media for 18.5 hours at 25 °C or 37 °C followed by labeling with 10 μg/ml $^{13}C_6$-pABA for 5 hours at 25 °C or 37 °C. At 37 °C, there was a two-fold decrease in *de novo* synthesized $^{13}C_6$-Q_6 in WT, but there was no significant change of ^{12}C-Q_6. In contrast, there was a marked decrease of both ^{12}C-Q_6 and $^{13}C_6$-Q_6 in *Δcoq9*:TS19 at the non-permissive temperature (Fig. 7A). Therefore, both the content of ^{12}C-Q_6 and the synthesis of *de novo* $^{13}C_6$-Q_6 are dramatically decreased by high-temperature incubation in the *Δcoq9*:TS19 mutant.

The nitrogen-containing compounds, 4-AP and IDMQ$_6$, accumulated in the *Δcoq9:TS19* mutant. For example, ^{12}C- and $^{13}C_6$-4-AP were uniquely present in the *Δcoq9:TS19* mutant at 37 °C (Fig. 7B) and levels of $^{13}C_6$-IDMQ$_6$ and $^{12}C_6$-IDMQ$_6$ increased six- and two-fold, respectively, at the non-permissive temperature (Fig. 7C). In contrast, WT had decreased amount of $^{13}C_6$-IDMQ$_6$ at the non-permissive temperature (Fig. 7C). Because Coq9 appears to be required to convert IDMQ$_6$ to DMQ$_6$, we also measured the amount of DMQ$_6$. We found that both $^{12}C_6$- and $^{13}C_6$-DMQ$_6$ were increased significantly in WT at the non-permissive temperature. However, the amount of $^{13}C_6$-DMQ$_6$ was not changed in the *Δcoq9:TS19* mutant at 37 °C (Fig. 7D). In conclusion, incubation of the *Δcoq9*:TS19 mutant at 37 °C led to a dramatic decline in the amount of Q_6 and accumulation of the nitrogen-containing compounds, 4-AP and IDMQ$_6$, that derive from $^{13}C_6$-pABA.

4. Discussion

Q plays a crucial role in mitochondrial electron transport and also serves as an important lipid-soluble antioxidant. Despite the obvious importance of Q in human health and mitochondrial disease, many questions remain regarding its biosynthesis. Although the Coq9 polypeptide is one of eleven polypeptides essential for Q biosynthesis in yeast and human cells, the functional role Coq9 plays in the Q biosynthetic pathway remains an outstanding question.

In this study, we examined the role of Coq9 in removing amino/imino groups from yeast Q intermediates derived from pABA (Fig. 1).

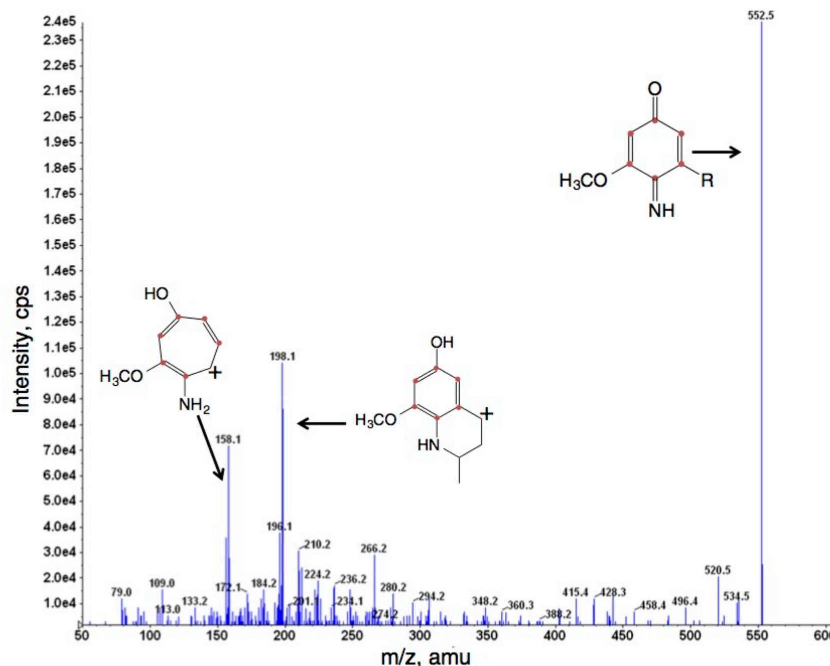

Fig. 3. Identification of *de novo* imino-demethyl-demethoxy-Q_6 ($^{13}C_6$-IDDMQ$_6$). Yeast *coq5-5 coq9Δ:mcCOQ8* was cultured and labeled with 50 μg/ml $^{13}C_6$ pABA as described in Fig. 2. Total lipids were extracted and analyzed by RP-HPLC-MS/MS. The fragmentation spectra are shown for $^{13}C_6$-IDDMQ$_6$ $[M+H]^+$ precursor ion ($^{13}C_6^{12}C_{31}H_{56}NO_2^+$; monoisotopic mass 552.4), the $^{13}C_6$-IDDMQ$_6$ tropylium ion $[M]^+$ ($^{13}C_6^{12}C_2H_{10}NO_2^+$; 158.1), and the $^{13}C_6$- IDDMQ$_6$ chromenylium ion $[M]^+$ ($^{13}C_6^{12}C_5H_{14}NO_2^+$; 198.1).

The finding that IDMQ$_6$ accumulates in a yeast *coq9* null mutant over-expressing *COQ8* [10] suggested that Coq9 is required for the deamination of IDMQ$_6$. Here we identified an earlier and new imino-intermediate in the pathway, IDDMQ$_6$. We discovered this intermediate when the *coq5-5* point mutant, defective in the *C*-methyltransferase step, was fed $^{13}C_6$-pABA. We showed that the *coq5-5* mutant fed $^{13}C_6$-pABA accumulated both $^{13}C_6$-DDMQ$_6$ and $^{13}C_6$-IDDMQ$_6$ (Fig. 2A and B). We speculated that the deamination of IDDMQ$_6$ would depend on Coq9. Therefore, we analyzed the intermediates that accumulated in the *coq5-5*, *Δcoq9* double mutant over-expressing Coq8 (*coq5-5 Δcoq9:mcCOQ8*). We found that this yeast strain lacked $^{13}C_6$-DDMQ$_6$, but still accumulated $^{13}C_6$-IDDMQ$_6$ (Fig. 2A and B). The data are consistent with the idea that Coq9 is required for removal of the nitrogen substituent for IDDMQ$_6$ to form DDMQ$_6$. The results also suggest that normally, the Coq5 *C*-methyltransferase acts prior to Coq9 and methylates IDDMQ$_6$ to form IDMQ$_6$. In the event that Coq5 activity is slow (or defective), then Coq9 is able to process IDDMQ$_6$ to DDMQ$_6$. There are likely to be profound differences between the yeast and human enzymes at these steps, because human cells are unable to convert pABA to Q [17].

Yeast Coq9 also plays an important role in supporting the activity of Coq6. This is evident because the dysfunction of Coq9 leads to the accumulation of 4-AP (Figs. 2C and 7B), which is an intermediate found in *coq6* null yeast mutants over-expressing *COQ8* [10]. In both the *Δcoq6* and *Δcoq9* mutants, the accumulation of $^{13}C_6$-4-AP depended on the presence of $^{13}C_6$-pABA and on Coq8 over-expression. Thus, even though Coq9 appears to be essential in removing the nitrogen groups from Q intermediates, we have not demonstrated that Coq9 is the enzyme that catalyzes the deamination step directly. Indeed, based on the accumulation of 4-AP, it is likely that Coq6 may play an important role in mediating the deamination step(s). It is important to note that there is some Coq6 activity present in the *coq9* null mutant over-expressing Coq8, because Q intermediates accumulate that harbor the Coq6-mediated hydroxyl group, such as $^{13}C_6$-IDMQ$_6$. While we have postulated potential pathways linking 4-AP, IDDMQ$_6$, and IDMQ$_6$ to the production of Q_6 (Fig. 1 and [7]), none have yet been proven to be productive intermediates in the pathway leading to Q_6.

While yeast Coq6 does not function very well in the absence of Coq9, several lines of evidence suggest that Coq7 is completely inactive in the absence of Coq9. Coq7 is a di-iron-containing hydroxylase that catalyzes the hydroxylation of DMQ [30,31]. Mutations in *COQ7* result in the accumulation of DMQ in *S. cerevisiae*, *C. elegans*, and mice [10,32,33]. Yeast *coq7* and *coq9* null mutants over-expressing *COQ8* both accumulate DMQ$_6$ when 4-HB is provided as the ring precursor [10]. In the yeast *Δcoq9* strain, Coq8 over-expression only slightly increases the steady-state level of Coq7, while Coq8 over-expression restored Coq9 polypeptide to wild-type level in *Δcoq7*. Purification of HA-tagged yeast Coq9 captures the yeast Coq7, Coq4, Coq6, and Coq5 polypeptides [6]. In addition, purification of tagged forms of Coq3 and Coq6 also capture Coq4, Coq5, Coq7, Coq8, and Coq9 [8]. These results indicate that yeast Coq9 and Coq7 are in a complex, together with other polypeptides and Q and Q intermediates, termed the CoQ-synthome [7,8]. Such Coq polypeptide biosynthetic complexes also appear to play a role in Q biosynthesis in the mouse. The lack of a functional Coq9 protein in homozygous Coq9 mutant mice causes a severe reduction in the Coq7 protein and accumulation of DMQ$_9$ [34]. Human cells with Coq9 defects accumulate an intermediate slightly more polar than Q_{10}; based on studies in yeast and mice, this seems likely to be DMQ$_{10}$ [35]. Thus it seems likely that the function of Coq9 in enhancing Coq7 function is conserved from yeast to humans.

To gain further insight into the function of Coq9, we created a temperature-sensitive yeast *coq9* allele (*coq9-ts19*). We showed that

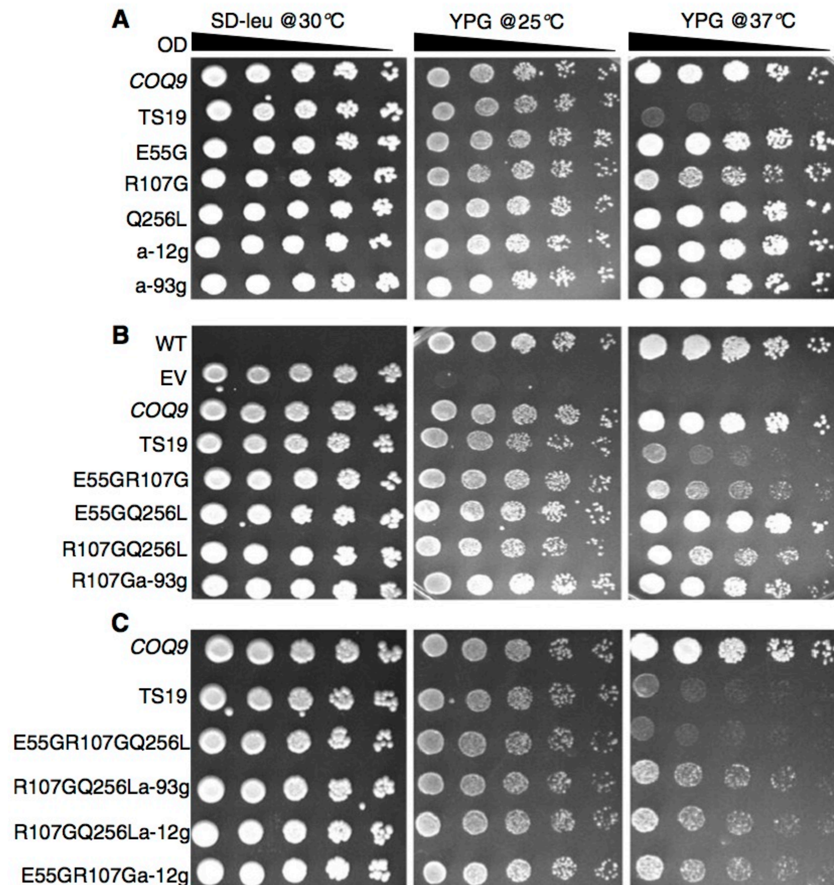

Fig. 4. Characterization of the mutations responsible for temperature sensitivity of the Coq9-TS19 polypeptide. The yeast *coq9* null mutant (W303ΔCOQ9) was transformed with plasmids containing the designated *coq9* mutations. pRS315 empty vector (EV), and yeast wild-type *COQ9* in pRS315 (*COQ9*), were included as controls. Yeast wild-type W3031B and the transformants were cultured overnight at 30 °C in YPD or SD−Leu media, respectively. Cell cultures were diluted to 0.2 based on A_{600nm} readings and 2 μl of 1:5 serial dilutions were spotted onto SD−Leu and YPG plate media and incubated at the specified temperatures for 3 days.

amino acid substitution mutations E55G, R107G, and Q256L recapitulated the YPG growth phenotype of the *coq9-ts19* mutant. At non-permissive temperature (37 °C), the presence of the Coq9-ts19 polypeptide led to a trend of decreased steady-state polypeptide levels of Coq4, Coq5, Coq6, and Coq7 in isolated mitochondria (Fig. 5). These observed changes in Coq polypeptide levels did not result from changes in the corresponding *COQ* RNA levels (Fig. 6). Lipid extracts prepared from the *coq9-ts19* mutant grown at non-permissive temperature showed a drastic decrease in Q_6 content and the accumulation of intermediates containing the nitrogen group, IDMQ_6 and 4-AP (Fig. 7). Taken together, these findings indicate that at the restrictive temperature, the *coq9-ts19* mutant loses the ability to support the activities catalyzed by Coq6 and Coq7, and that the CoQ-synthome is destabilized.

In contrast, when wild-type yeast cells were subjected to the same temperature shift, steady-state polypeptide levels of the Coq4, Coq7, and Coq6 polypeptides were increased (Fig. 5). Again, these changes were not paralleled by changes in the corresponding *COQ* RNA levels (Fig. 6). In contrast to the increase in the Coq polypeptide content at high temperature, the level of *de novo* synthesized Q_6 was decreased, while the level of *de novo* synthesized $^{13}C_6$-DMQ_6 increased (Fig. 7). This observation is consistent with impaired Coq7 function at the elevated temperature. The phosphorylation state of Coq7 affects Q_6 biosynthesis and the status of respiratory metabolism can cause Coq7 to become dephosphorylated or phosphorylated [36]. It would be interesting to compare the phosphorylation state of Coq7 at permissive and non-permissive temperatures. It is also possible that certain Coq enzyme activities may be sensitive to high temperatures. It was shown that Coq3 homologs from either *C. elegans* or *S. cerevisiae* rescue the *E. coli ubiG* mutant at 30 °C, but not at 37 °C [37].

It is important to note that the recent study by Lohman et al. [38] provides insights into the possible effects of these yeast Coq9-ts19 amino acid substitutions. Lohman et al. solved the structure of human Coq9 and identified it as a member of an ancient protein family TFR (TetR family of regulators) with a canonical amino terminal helix-turn-helix (HTH) domain. Two human Coq9 polypeptides crystallized as a dimer and formed a hydrophobic interface that binds lipids, including phospholipids and Q [38]. Another separate surface patch of Coq9 was shown to be key to the ability to bind human Coq7. Intriguingly, the authors identified key amino acid residues in the human Coq9 polypeptide that affected binding with Coq7. We used the human Coq9

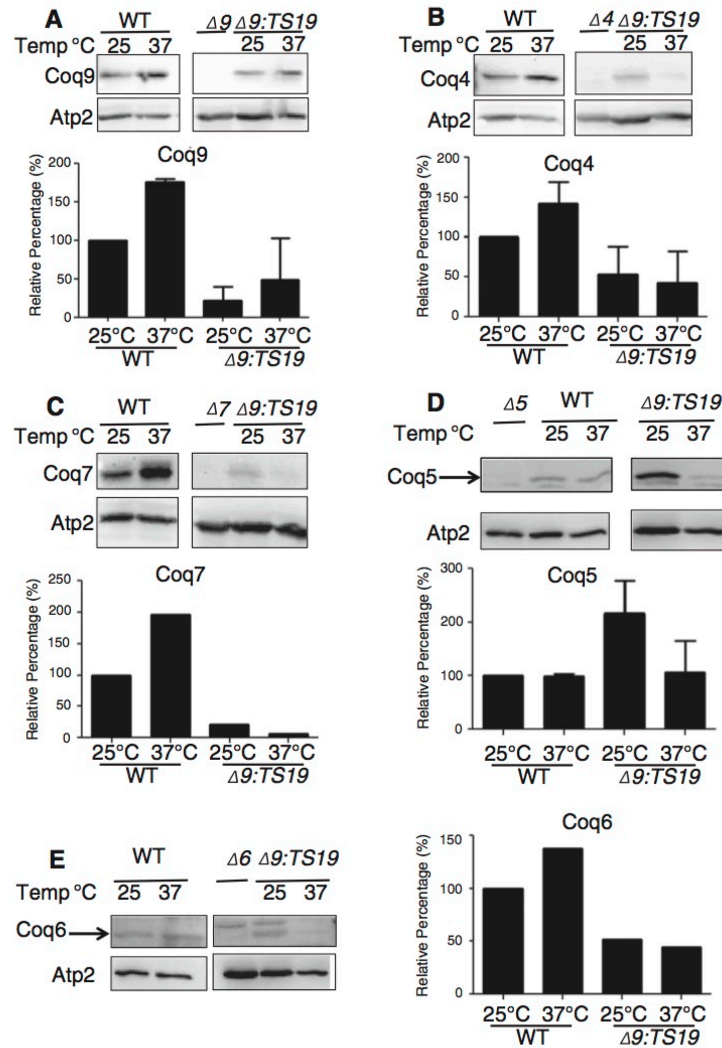

Fig. 5. Expression of Coq9-ts19 polypeptide affects steady-state levels of other yeast Coq polypeptides in response to different growth temperatures. The mitochondria of W3031B (WT) and W303ΔCOQ9 harboring the temperature-sensitive plasmid TS19 (Δ9:TS19) were isolated after yeast were grown for 18.5 hours at either 25 °C or 37 °C. Mitochondria were also isolated from the null control strains BY4741Δcoq9 (Δ9), W303Δcoq7 (Δ7), W303Δcoq4 (Δ4), W303Δcoq5 (Δ5), and W303Δcoq6 (Δ6) after yeast were grown overnight at 30 °C. Purified mitochondria (15 μg protein) were separated by SDS–PAGE and analyzed by immunoblot. Immunoblots were performed with antibodies against the designated polypeptides: Coq4, Coq5, Coq6, Coq7, Coq9, and Atp2. Images presented within a given panel were derived from the same gel, and thus the band intensities corresponding to the same antisera can be directly compared. Densitometry quantification was conducted with the software ImageJ (NIH) and plotted as graphs. The signals of Coq4, Coq5, Coq6, Coq7, and Coq9 were each normalized to the signals of Atp2. Normalized values were then compared to WT at 25 °C to get the relative percentage. The immunoblot depicted is representative of two independent blots performed with two different preparations of purified mitochondria. (*A*, *B*, *D*) Bars designate the average signal + S.D. (n=2); (*C*, *E*) Each bar represents one measurement (n=1) because signals for Coq6 and Coq7 were detectable at 37 °C in only one set of the immunoblots.

structure to predict the structure of yeast Coq9 with the protein homology/analogy recognition engine (Phyre 2) [39]. The predicted yeast structure is comprised of residues P40 to L231, so only the E55G and R107G can be evaluated (Fig. 8A). In the predicted structure, residue E55 is at the end of α helix one and R107 is in α helix five (Fig. 8A). When compared to the human Coq9 structure, yeast E55 corresponds to human A113, which resides at the C-terminus of α helix one and is part of the HTH domain (Fig. 8B). The HTH motif is structurally similar to the TFR family of bacterial transcriptional regulators but is predicted to lack DNA-binding capacity [38]. The presence of the E55G on its own did not affect growth on at the non-permissive temperature (Fig. 4). This is similar to the observation that single mutations introduced into the HTH domain of yeast Coq9 did not affect respiration competence [38]. Yeast R107 aligns with Q165 in α helix four of human Coq9 (Fig. 8B). We found that the R107G substitution on its own produced a moderate temperature-sensitive phenotype, but the combination or

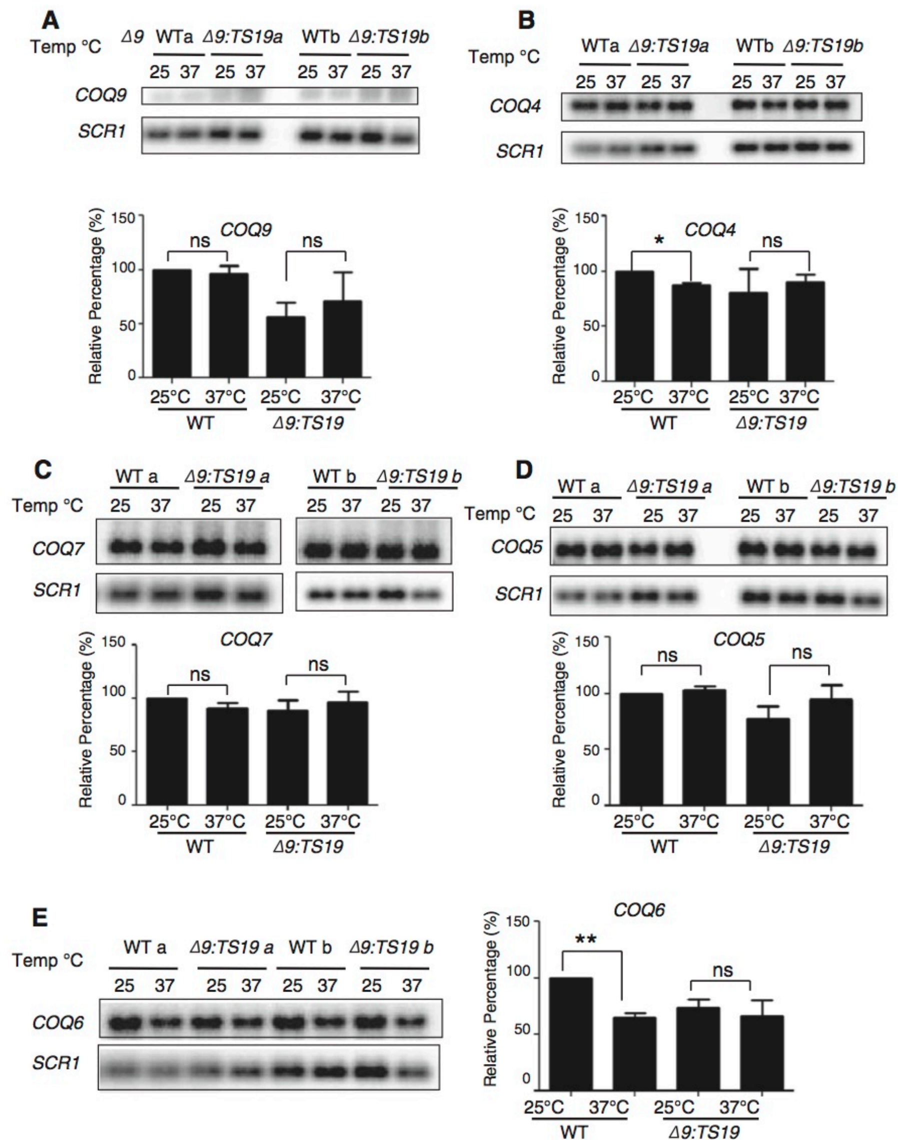

Fig. 6. The observed changes in Coq polypeptide levels with temperature do not correspond to alterations in *COQ* RNA content. The total RNA of W3031B (WT) and W303Δcoq9 harboring the temperature-sensitive plasmid TS19 (Δ9:TS19) were extracted after yeast were grown for 18.5 hours at 25 °C or 37 °C. Aliquots of RNA (5 μg) were separated by 1.2% agarose gel and analyzed by Northern blot. Hybridizations were performed with probes against the designated RNA: *COQ4*, *COQ5*, *COQ6*, *COQ7*, *COQ9*, and *SCR1*. Northern blot assay signals were quantified with the Quantity One software from the Bio-Rad FX Plus Phosphorimaging System. The quantified signals of *COQ4*, *COQ5*, *COQ6*, *COQ7*, and *COQ9* were each normalized by the signals of *SCR1*. Normalized values were then compared to WT at 25 °C to get the relative percentage. Each bar represents a total of two measurements from two independent samples (n= 2). Significant changes in the amounts of mRNA at different temperatures were determined with the Student's two-tailed *t*-test. The *symbols represent mRNA in samples at 37 °C compared to mRNA at 25 °C; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

either E55G or Q256L in combination with R107G gave a much more pronounced temperature sensitivity. In the human Coq9 structure, Q165 resides in a region that is neither highly conserved nor is shown to be important in lipid binding or interaction with human Coq7 [38]. Our immunoblot analyses show that at non-permissive temperature, the steady-state level of Coq9-ts19 is increased, while other Coq polypeptides are destabilized (Fig. 5A).

According to a model proposed by Gonzalez-Mariscal, Coq7 is recruited to the precomplex to catalyze the conversion of DMQ_6 to Q_6 [40]. They proposed that Coq9 plays an important structural role to

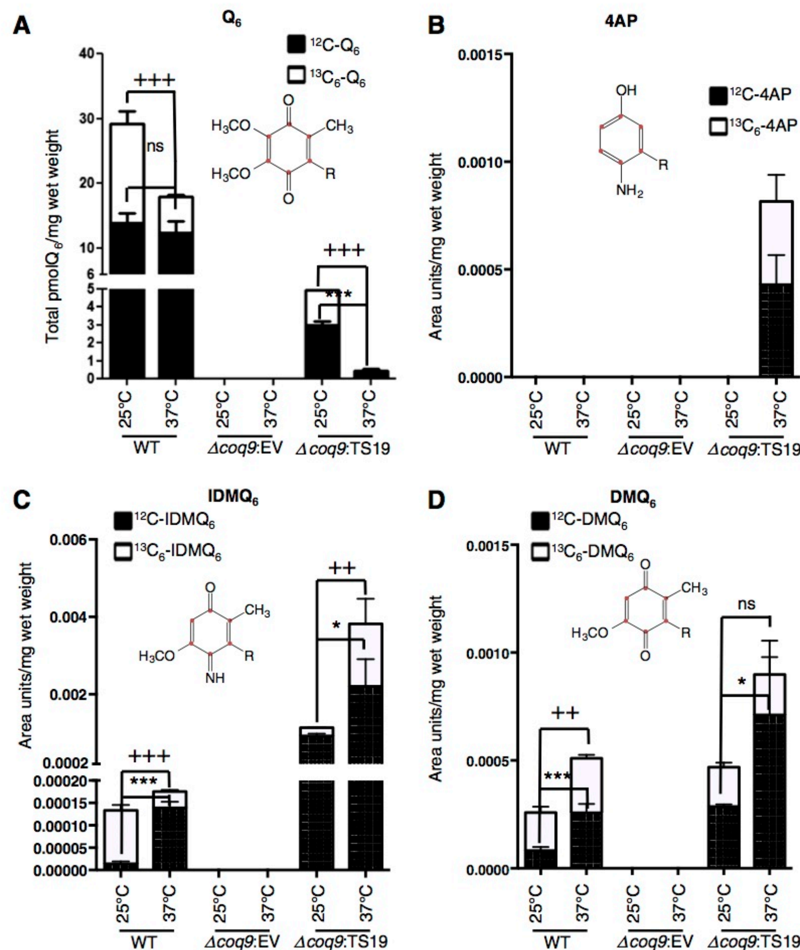

Fig. 7. Yeast strains expressing Coq9-ts19 have decreased Q_6 content and accumulate higher levels of nitrogen-containing Q intermediates at the restrictive temperature. Yeast W3031B (WT), W303ΔCOQ9 harboring pRS315 empty vector (Δcoq9: EV), and W303ΔCOQ9 harboring the temperature-sensitive plasmid TS19 (Δcoq9: TS19) were seeded in 20 ml of SD-Complete (WT) or SD−Leu (Δcoq9: EV and Δcoq9: TS19) at 0.01 A_{600nm}/ml and grown for 18.5 hours at 25 °C or 37 °C. $^{13}C_6$-pABA (10 μg/ml) was added to yeast cultures and incubations were continued at either 25 °C or 37 °C. After labeling for 5 hours, yeast cells (30 A_{600nm}) were collected as cell pellets from which lipids were extracted and analyzed by RP-HPLC-MS/MS. Each bar represents a total of four measurements from two independent samples each with two injections. Black bars represent the amount of the designated ^{12}C-compounds and white bars represent the amount of $^{13}C_6$-compounds. For Q_6, $IDMQ_6$, and DMQ_6, the total amounts of the ^{12}C- and $^{13}C_6$-compounds represent the sum of reduced and oxidized forms; ^{12}C-AP and $^{13}C_6$-4AP, were present only in the reduced form. Significant changes in the amounts of Q_6 and Q_6 intermediates at different temperatures were determined with the Student's two-tailed *t*-test. The *symbols represent ^{12}C-compounds in samples at 37 °C compared to ^{12}C-compounds at 25 °C; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The + symbols represent $^{13}C_6$-labeled compounds in samples at 37°C were compared to samples at 25 °C; +, $p < 0.05$, ++, $p < 0.01$, +++, $p < 0.001$; *ns* designates "non-significant".

stabilize the Q-biosynthetic complex and is recruited to form a 700 kDa precomplex as part of the nucleation process initiated by Coq4 binding to HHB [40]. Hence it seems plausible that the temperature-sensitive mutations cause misfolding of yeast Coq9-ts19 at high temperature and prevent its proper function or interaction with other Coq polypeptides and lead to the destabilization of the precomplex. There might be a small amount of functional Coq9-ts19, so some of the 700 kDa precomplex is able to form and produces DMQ_6 [40]. Although their model did not depict the interaction of Coq9 and Coq7 as the means of Coq7's recruitment, the results presented here, and the Coq9 structure by Lohman et al. suggest that this may be the case. There is a large amount of DMQ_6 accumulated in the temperature-sensitive mutant at non-permissive temperature, but very little Q_6 was produced (Fig. 7). In this scenario, the mutations in Coq9-ts19 disrupt the interaction of Coq7 and Coq9, so Coq7 cannot bind to the pre-complex and perform its function. Lohman et al. [38] noted that several residues predicted to affect the interaction of yeast Coq9 with Coq7 resulted in decreased Q_6 and increased DMQ_6. It would be interesting to determine the effect of these mutations on the accumulation of $IDMQ_6$.

In conclusion, we found that yeast Coq9 is required for the deamination of 4-AP, $IDDMQ_6$, and $IDMQ_6$. At the non-permissive temperature, the coq9-ts19 mutant has low steady-state levels of Coq4, Coq5, Coq6, and Coq7 polypeptides, shows defective growth on non-fermentable carbon source and a drastic decrease in the content of Q_6, and accumulates imino-/amino-Q intermediates The results presented here identify Coq9 as a multi-functional protein that is required for the function of

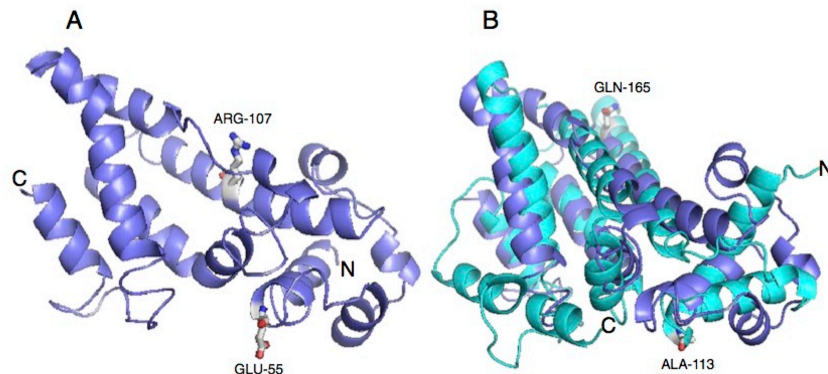

Fig. 8. A homology model of yeast Coq9. (*A*) The yeast Coq9 structure was predicted based on the crystal structure of human Coq9 by protein homology/analogy recognition engine (Phyre 2). There are 163 aligned residues, 13% identity and 99.54% confidence. The positions of two of the temperature-sensitive amino acid substitution mutations are shown: E55 in α helix one, and R107 in α helix five. (*B*) The predicted yeast Coq9 structure (purple) is shown aligned with human Coq9 structure (cyan) [38]. Yeast E55 corresponds to human A113 (both are in a helix one in the HTH domain) and yeast R107 aligns with Q165 in α helix four of human Coq9.

Coq6 and Coq7, for removal of the nitrogen substituent from pABA-derived Q intermediates, and is an essential component of the CoQ synthome.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

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Chapter 4

Human COQ9 rescues a *coq9* yeast mutant by enhancing Q biosynthesis from 4-hydroxybenzoic acid and stabilizing the CoQ-synthome

Abstract

Coq9 is required for the stability of a mitochondrial multi-subunit complex, termed the CoQ-synthome, and the deamination step of Q intermediates that derive from para-aminobenzoic acid (pABA) in yeast. In human, mutations in the *COQ9* gene cause neonatal-onset primary Q_{10} deficiency. In this study, we examined human Coq9's complementation of yeast *coq9* point and null mutants. We found that expression of human *COQ9* rescues the growth of the temperature-sensitive yeast mutant, *coq9-ts19*, on a non-fermentable carbon source and increases the content of Q_6, by enhancing Q biosynthesis from 4-hydroxybenzoic acid (4HB). To study the mechanism for the rescue by human Coq9, we study the steady-state levels of yeast coq polypeptides in the mitochondria of temperature-sensitive yeast mutant expressing the human *COQ9*. With SDS-PAGE and Western blot, we showed that the expression of human *COQ9* significantly increased the levels of yeast Coq4, Coq6, Coq7 and Coq9 at permissive temperature and human Coq9 was destabilized at non-permissive temperature. Next, we showed that a small amount of the human Coq9 co-purified with tagged Coq6, Coq6-CNAP, indicating that human Coq9 interacts with the yeast Q-biosynthetic complex. We concluded that human Coq9 rescues yeast *coq9* point mutant by stabilizing the CoQ-synthome and increasing Q biosynthesis from 4HB. This finding provides a powerful approach to study the function of human Coq9 using yeast as a model.

Introduction

Coenzyme Q (Q) is a lipid that functions as an electron and proton carrier in the mitochondrial respiratory chain and as a lipid-soluble antioxidant (1). Q is composed of a redox active benzoquinone ring and a polyisoprenoid side chain, which contains six isoprenic units in *Saccharomyces cerevisiae* (Q_6) and ten isoprenic units in humans (Q_{10}) (2). Both yeast and human cells are able to use 4-hydroxybenzoic acid (4HB), resveratrol and coumarate to make Q (3). However, while para-aminobenzoic acid (pABA) is a ring precursor for Q in yeast (4), mammalian cells were not able to synthesize Q from pABA (3). The biosynthetic pathway of Q is highly conserved among species. Genes involved in Q biosynthesis in yeast include *COQ1-10, YAH1, ARH1, ADCK1* and *ADCK2*, all of which have human homologs (5). A new gene involved in yeast Q biosynthesis was recently identified as *COQ*11 (6), and whether it has a human homolog requires further investigation. A good number of human homologs can rescue the corresponding yeast COQ mutants: *COQ2, COQ3, COQ4, COQ6, COQ7, ADCK3, ADCK4, COQ10A, COQ10B* (5) and *COQ5* (7).

Yeast Coq9 is required for Q_6 biosynthesis and the stability of the CoQ synthome (8,9). Its function is not fully understood, but it is required for the deamination of nitrogen substituent-containing Q intermediates derived from pABA. For example, imino-demethoxy-Q_6 ($IDMQ_6$) and 3-hexaprenyl-4-aminophenol (4-AP) accumulate in *coq9* null mutant when *COQ8* is over-expressed to stabilize the Q-biosynthetic complex and pABA is provided as the precursor (10). In yeast *coq5* point mutant, *coq5-5*, demethyl-demethoxy Q_6 ($DDMQ_6$) and imino-demethyl-demethoxy Q_6 ($IDDMQ_6$) accumulate (11), but only $IDDMQ_6$ accumulates when *COQ9* is knocked out in *coq5-5* (12). We generated a *coq9* temperature sensitive mutant, *coq9-ts19* (TS19) that contains the following point mutations: Adenine−12→Guanine (a−12g),

Adenine−93→Guanine (a−93g), Glu55→Gly (E55G), Arg107→Gly (R107G), and Gln256→Leu (Q256L). We found that at non-permissive temperature, the levels of Coq9-ts19 increased, but other yeast Coq polypeptides, Coq4, Coq5, Coq6, and Coq7 decreased and nitrogen-containing intermediates accumulated when pABA is provided (12). Therefore, yeast Coq9 controls the removal of nitrogen group of Q intermediates derived from pABA. Interestingly, human cells cannot synthesized Q from pABA, indicating human Coq9 has a different role in coenzyme Q biosynthesis.

The human COQ9 homolog is required for Q_{10} biosynthesis; a mutation was identified that caused neonatal-onset primary Q_{10} deficiency (13). A patient with a homozygous nonsense mutation in the *COQ9* gene (Arg_{244}STOP) presented with neonatal lactic acidosis and later developed multisystem disease including intractable seizures, global developmental delay, hypertrophic cardiomyopathy, and renal tubular dysfunction. Cultured skin fibroblasts from the patient were examined and found to contain low levels of Q_{10} relative to control subjects and a compound slightly more polar than Q_{10}, suggestive of a Q_{10}-intermediate (13). Garcia-Corzo *et al*. generated a *Coq9* mutant mouse by introducing a R239X mutation that recapitulates the R244STOP human *coq9* mutation (14). The $Coq9^{X/X}$ mice showed histologic and behavioral signs that mirrored mitochondrial encephalomyopathy associated with primary Q deficiency in human patients. A widespread Q deficiency was noted in these mice along with a dramatic reduction in the steady state level of the COQ7 polypeptide and accumulation of demethoxy-Q_9 (DMQ_9) (14). A resent study solved the crystal structure of human Coq9 (15). They showed that human Coq9 functions as a dimer and it has a hydrophobic interface that binds lipids and a surface patch that binds human Coq7 (15). Taken together the results suggest that the Coq9 polypeptide is required for Coq7 function in Q biosynthesis.

Yeast has been a great model for the studies of Q biosynthesis and it can be a powerful system to study human Coq proteins with unknown functions. In most cases, human *COQ* homologs are able to rescue the corresponding yeast *coq* mutants (16,17). For example, human COQ6 expressed from a plasmid with yeast mitochondrial leader rescued the yeast *coq6* null mutant for growth on a non-fermentable carbon source (18); human COQ5 with its first 55 amino acids replaced by the first 54 amino acids of yeast Coq5 restored growth on medium containing a non-fermentable carbon source and Q_6 content of a yeast *coq5* null mutant over-expressing *COQ8* (11). However, expression of human COQ9 in yeast did not restore Q biosynthesis in yeast *coq9* mutants (13,17). In this study, we tested whether human COQ9 could rescue distinct yeast *coq9* mutants. The results presented indicate that under certain conditions human COQ9 functions to restore yeast Q biosynthesis, but that the potential of yeast Coq9 to remove amino/imino groups from Q-intermediates is a functional role that is not shared with human COQ9.

Materials and Methods

Yeast strains and growth media

S. cerevisiae strains used in this study are listed in table 1. Growth media used in this study included: YPD (2% glucose, 1% yeast extract, 2% peptone), YPGal (1% yeast extract, 2% peptone, 2% galactose, 0.1% dextrose), and YPG (3% glycerol, 1% yeast extract, 2% peptone). They were prepared as described (19). Synthetic Dextrose/Minimal medium consisted of 0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, and amino acids were added to final concentrations as described (20). Selective SD/Minimal medium lacking uracil (SD–Ura) and selective SD/Minimal medium lacking uracil and leucine (SD–Ura–Leu) were similarly prepared. Agar plate media were prepared as described above and included 2% bacto agar (Fisher).

Construction of plasmids

Coq8 was over-expressed in yeast with the p4HN4 plasmid (mc*COQ8*). The *COQ8* gene was cloned in pRS426, a multi-copy yeast shuttle vector, resulting mc*COQ8* (21). To construct plasmids expressing human *COQ9*, we cloned human *COQ9* into pQM (22) and pRCM (23). These are respectively low- and multi-copy vectors that express ORFs fused to the yeast Coq3 amino terminal mitochondrial leader sequence (amino acids 1-34) and under control of the yeast *CYC1* promoter. Human *COQ9* was amplified from pBGcoq9, which contains the human *COQ9* ORF in YEpJB1-21-10 and expressed from a constitutive PGK promoter (13). The human *COQ9* ORF was amplified with Taq polymerase and primers Hcoq9F (5'-ATCGATATGGCGGCGG CGGCGGTAT-3' with ClaI restriction site at the 5' end) and HcoqR (5'-GGTACCTC ACCGACGCTGGTTTAGACCTGTCAAGTTCTTGAGC-3' with KpnI restriction site at the 5' end). PCR products were inserted into the TOPO vector resulting in a plasmid named

HCOQ9TOPO. HCOQ9TOPO was digested with the restriction digestion enzymes ClaI and KpnI (New England BioLabs) and inserted in pQM or pRCM prepared with ClaI and KpnI, resulting in the plasmids scHCOQ9 and mcHCOQ9, respectively. The nucleotide sequence of the human *COQ9* ORF in scHCOQ9 and mcHCOQ9 was confirmed by sequencing (UCLA sequencing core, Los Angeles).

Disruption of COQ9 in W3031B yeast strain

A PCR product containing the KanMX4 gene was amplified with the genomic DNA isolated from BY4741Δ*coq9* (used as template) and with primers that annealed to 100 bp upstream and downstream of the *COQ9* ORF. The sequences of the primers utilized were: 5'-TTTGGGCCTACATAAGGTACTTC-3' and 5'-CGCACAGACCAATAAATCTGCC-3'. The PCR product was then transformed into the yeast W3031B to create W303Δcoq9K. Transformants that grew on YPD + 200 μg/ml G418 (Geneticin) were selected. Proteins were extracted from these transformants as described (24) and separated by SDS-PAGE with a 10% polyacrylamide gel. Proteins were transferred to an Immobilon-P transfer membrane (Millipore) and analyzed by immunoblotting as described (8). The primary antibody against Coq9 was used at a 1:1000 dilution and the secondary antibody, goat anti-rabbit IgG H&L chain specific peroxidase conjugate (Calbiochem), at a 1:10,000 dilution. The absence of Coq9 polypeptide confirmed that *COQ9* was replaced with KanMX4. The resulting null mutant is W303Δcoq9K.

Lipid extraction and detection of Q_6-intermediates by HPLC and tandem mass spectrometry

The *de novo* synthesis of Q_6 and Q_6-intermediates was tracked in yeast cells labeled with $^{13}C_6$-pABA or $^{13}C_6$ 4HB followed by lipid analysis. Labeling media were prepared with 10 μg/ml $^{13}C_6$-pABA or $^{13}C_6$-4HB dissolved in ethanol (0.2% final concentration). Cells were collected (a

total of 50 A_{600nm}) as pellets by centrifugation after 12.5 hours of labeling. Q_4 was added (164 pmol) to each cell pellet to serve as an internal standard. Lipid extracts were analyzed by RP-HPLC-MS/MS (10). For liquid chromatography, a phenyl-hexyl column (Luna 5u, 100 × 4.60 mm, 5-µm, Phenomenex) was used. The mobile phase has Solvent A (methanol/isopropanol, 95:5, with 2.5 mM ammonium formate) and Solvent B (isopropanol, 2.5 mM ammonium formate). Solvent B was increased linearly from 0 to 5% with the flow rate increased from 600 to 800 µl/min from 0 to 6 min. The flow rate and mobile phase were changed back to 600 µl/min and 100% Solvent A respectively at 7 min. Multiple reaction monitoring mode (MRM) analysis was performed with the 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA). Data were processed with Analyst version 1.4.2 software (Applied Biosystems).

To quantify Q_6 content, the peak areas of $^{12}C_6$-Q_6 (sum of oxidized and reduced) and $^{13}C_6$-Q_6 (sum of oxidized and reduced) were normalized by the peak areas of Q_4 (sum of oxidized and reduced); the pmol amounts were then determined from the Q_6 standard curve. The pmol of ^{12}C-Q_6 and $^{13}C_6$- Q_6 were further normalized by the wet weight of yeast pellets. Chemical standard for DMQ_6 is not available. To quantify this intermediate, the peak areas (sum of oxidized and reduced DMQ_6) were normalized by the recovery of Q_4 (sum of oxidized and reduced peaks). Finally, calculated values were further normalized by the wet weight of yeast pellets.

Mitochondrial isolation and immunoblot analyses with temperature-sensitive mutants expressing the human COQ9 homolog

Mitochondria were isolated from yeast cells and analyzed by SDS-PAGE followed by Western blot. Yeast cultures were grown to 3-4 A_{600nm} in YPGal medium at different temperatures (W3031B, W303Δ*coq9*:TS19, and W303Δ*coq9*:TS19+mcHCOQ9 were grown at

25 °C and 37 °C for 18.5 hours; BY4741ΔCOQ9, W303ΔCOQ7, W303ΔCOQ4, W303ΔCOQ6 were grown at 30 °C overnight). Crude mitochondria were isolated from 1 L of culture as described (25). Mitochondria were further purified with an OptiPrep discontinuous iodixanol gradient as described (8). The bicinchoninic acid assay was used to measure the total protein concentration in purified mitochondria (Life Technologies). Purified mitochondria were solubilized with digitonin as described (8), and 15 μg of mitochondria were separated by SDS-PAGE with 10% polyacrylamide gels. Proteins were transferred to Immobilon-P transfer membranes (Millipore) and immunoblot analyses were performedas described (8). The source and use of primary antibodies is described in Table 2. Secondary antibodies were goat anti-rabbit IgG H&L chain specific peroxidase conjugate (Calbiochem), 1:10,000.

Immunoprecipitation of Coq6-CNAP expressing the human COQ9 homolog

Purified mitochondrial proteins (13mg) were solubilized with 4 mg digitonin /ml as described above. The soluble digitonin extract was collected after 100,000 × *g* centrifugation (Optimax TLX). Immunoprecipitation was then performed on the solubilized mitochondria with Ni-NTA resin as described in (6). Briefly, Ni-NTA resin (800 μl bed volume) was equilibrated with two volumes of lysis buffer. Solubilized mitochondria and 8 ml of lysis buffer were added to 800 μl of pre-equilibrated Ni-NTA resin (bed volume) in a 15 ml falcon tube and rotated for 1.5 hours at 4°C. The flow-through was then collected with a flowthrough column. Resin was subjected to two washes with Ni-NTA wash buffer (W1 and W2) and eluate fraction (E) from immunoprecipitation were collected. Aliquots of each fraction were examined for presence of Coq polypeptides: 0.17% of the FT, 0.25% of W1, 0.25% of W2, 1% of E1, 0.5% of E2, and 1.25% of Ni-NTA resin were analyzed by SDS-PAGE followed by immunoblot with anti-bodies

against Coq9, human Coq9 (1:1000), Coq6, 1:250 and Atp2, 1:4000. Purified mitochondria (15 μg protein) from CNAP6: *mcHCOQ9* were included as control.

Results

Expression of a human COQ9 homolog rescues the growth of the *Δcoq9K:TS19 mutant on medium containing a nonfermentable carbon source*

Expression of human COQ9 has so far failed to rescue yeast *coq9* null mutant growth (13,17). It seemed likely that the destabilization of other Coq polypeptides in the yeast *coq9* null mutant might account for the inability of human *COQ9* to rescue. To stabilize other Coq polypeptides in *coq9* null mutant, we co-expressed multi-copy *COQ8* (*mcCOQ8*) with either single copy (*scHCOQ9*) or multi-copy of human *COQ9* (*mcCOQ9*) in a *coq9* null yeast mutant (W303Δ9K) and tested its growth on YPG plate medium, containing glycerol as the sole non-fermentable carbon source. However, none of the conditions tested enabled human COQ9 to rescue the growth of the *coq9* null mutant (Fig. 1).

Next, we turned to the *Δcoq9K:*TS19 mutant, which retains yeast Coq9 and other Coq polypeptide levels and is able to grow on YPG at the permissive temperature (25 °C) (12), but shows defective growth on YPG at the non-permissive temperature (37 °C ; Fig. 1). Expression of *mcCOQ8*, *scHCOQ9*, or *mcHCOQ9* were each able to rescue the growth of the *Δcoq9:*TS19 mutant on YPG at the non-permissive temperature. The rescue by *mcCOQ8* and *mcHCOQ9* is similar to the rescue by yeast wild-type *COQ9*, and more robust as compared to *scHCOQ9* (Fig. 1). At the permissive temperature it is not possible to distinguish the effects of single-copy human *COQ9* or multi-copy *COQ8*. The yeast Coq9-ts19 mutant still functions at 25 °C, and its growth is similar to wild type. However, there is a slight increase of growth of *Δcoq9K:*TS19 harboring *mcHCOQ9*. Yeast were also plated on SD−Ura−Leu to confirm that W303Δ9K was successfully transformed with the two plasmids. The empty vector pRS426, which is the parent vector of *mcCOQ8*, was included as a control (EV). As expected, *Δcoq9K:*TS19 cannot be

rescued by the empty vector (Fig.1). Therefore, the rescue effects were specific to expression of either human *COQ9* or over-expression of yeast *COQ8*.

*In the yeast Δcoq9K:*TS19 *mutant, expression of human COQ9 increased the de novo synthesis of Q_6 from 4HB and over-expression of Coq8 increased the de novo synthesis of Q_6 from pABA*

We found that Coq9 is required to remove the nitrogen group from Q_6 intermediates (12), so we compared the *de novo* synthesis of $^{13}C_6$-Q_6 from either $^{13}C_6$-pABA or $^{13}C_6$-4HB. The yeast W303ΔCOQ9K harboring TS19 was transformed with the designated plasmids and Q/Q intermediates levels were determined at permissive and non-permissive temperatures. The presence of mcCOQ8, significantly increased the amount of $^{13}C_6$-Q_6 synthesized from $^{13}C_6$-pABA at the permissive temperature relative to the empty vector control (Fig. 2A). At the non-permissive temperature, *mcCOQ8* and both the s*cHCOQ9* and *mcHCOQ9* plasmids increased the amount of $^{13}C_6$-Q_6 synthesized from $^{13}C_6$-pABA (Fig. 2A). In contrast, *mcCOQ8* and the human *COQ9* homolog increased the amount of $^{13}C_6$-Q_6 synthesized from $^{13}C_6$-4HB at the permissive temperature, but only the human *COQ9* homolog increased the amount of $^{13}C_6$-Q_6 synthesized from $^{13}C_6$-4HB at the non-permissive temperature (Fig. 2D). There is a significant increase of $^{13}C_6$-DMQ_6 in W303Δ9K:TS19 with the expression of *mcCOQ8* and both the s*cHCOQ9* and *mcHCOQ9* (Fig. 2B and 2E). Interestingly, *mcHCOQ9* has the most dramatic effect on $^{13}C_6$-DMQ_6 levels when $^{13}C_6$-4HB was provided at non-permissive temperature (Fig.2E). These findings suggest that expression of human COQ9 rescues the *Δcoq9K:*TS19 mutant by increasing Q biosynthesis with 4HB as the precursor. The levels of $^{12}C_6$-Q_6 were elevated by *mcCOQ8* and human *COQ9* at both permissive and non-permissive temperatures (Fig. 2C and F), perhaps indicating that *mcCOQ8* and human *COQ9* may enhance the utilization of other ring precursors to increase Q content.

*In the yeast Δcoq9K:*TS19 *mutant, expression of human COQ9 stabilizes yeast Coq polypeptides at permissive temperature*

To investigate how the expression of human *COQ9* homolog rescues the growth of the *Δcoq9K:*TS19 mutant on respiratory medium, we determine whether human Coq9 changes other Coq polypeptide levels. We grew wild type (WT), Δ*coq9K*:TS19 (Δ9K:TS19) and Δ9K:TS19+ *mcHCOQ9* yeast in YPGal for 18.5 hours at 25 °C or 37 °C, and then isolated mitochondria. The steady-state levels of Coq4, Coq6, Coq7, Coq9, and human Coq9 in purified mitochondria were analyzed by Western blotting (Fig. 3). The levels of Atp2 were analyzed as loading control. At non-permissive temperature, the expression of Coq9-ts19 causes destabilization of other yeast Coq polypeptides and human Coq9. In wild type, Coq4, Coq6, Coq7 and Coq9 levels were increased at 37 °C (Fig. 3). In the Δ*coq9K*:TS19 mutant, Coq9-ts19 levels were increased at 37 °C (Fig. 3A), while Coq4, Coq6, and Coq7 were decreased at 37 °C (Fig. 3). The changes of Coq polypeptides in either wild type or the temperature-sensitive mutant at different temperature did not result from changes in the corresponding *COQ* RNA levels (12). When human *COQ9* homolog was expressed in Δ*9K*:TS19 (Δ9K:TS19+ *mcHCOQ9*), the steady state levels of Coq4, Coq6, Coq7 and Coq9 were significantly increased at permissive temperature (Fig. 3). The results suggest that at permissive temperature the expression of human *COQ9* stabilizes certain yeast Coq polypeptide. Two bands were detected in the mitochondria of Δ*9K*:TS19+mcHCOQ9 by antibody against human Coq9. Based on the mass of the polypeptides, it seems likely that the top band corresponds to unprocessed human Coq9 with the mitochondrial leader (39 kDa), and the lower band is processed human Coq9 (30.5 kDa). At non-permissive temperature, human Coq9 was also destabilized (Fig. 3A). The destabilization is specific to human Coq9 and Coq proteins in the CoQ synthome because the steady state levels of Atp2, the beta subunit of the F1

sector of the mitochondrial F_1F_0 ATP synthase, did not change at higher temperature. It is possible that human Coq9 is associated with the CoQ synthome.

The human COQ9 polypeptide associates with the yeast Q-biosynthetic complex

To determine whether the human COQ9 polypeptide might interact with the CoQ synthome (8), we expressed human *COQ9* in the yeast strain Coq6-CNAP(CNAP6). We chose Coq6-CNAP as the bait protein because Coq9 is known to be important for the Coq6 hydroxylation step (10). A consecutive non-denaturing tag containing a His_{10} tag and protein C epitope was integrated at the C-terminus of yeast Coq6, resulting Coq6-CNAP (6). Mitochondria were isolated from CNAP6: *mcHCOQ9* after they were grown in YpGal overnight at 30°C. Isolated mitochondria were solubilized with digitonin and subjected to consecutive non-denaturing affinity chromatography. The CNAP6 has normal levels of Coq6 and Q_6 and co-precipitates other Coq polypeptides in the CoQ synthome (Coq4, Coq5, Coq7, Coq8 and Coq9) (6). Purified mitochondria of CNAP6:*mcHCOQ9* were solubilized and subjected to Ni-NTA resin. Fractions corresponding to flow through (FT), washes (W1 and W2), eluate (E1 and E2), and beads after elution were analyzed by SDS-PAGE and Western blot. We found that unprocessed human Coq9 and yeast Coq9 co-purified with Coq6-CNAP, (Fig. 6). As a negative control, we also blotted with antibody against Atp2. Atp2 did not co-purify with Coq6-CNAP (Fig. 6) as expected. The majority of human Coq9 was detected in the flow through and wash fractions, indicating the interaction between human Coq9 and the Coq6-containing complex is weak or only a small fraction of the over-expressed human Coq9 might be expected to interact with the yeast Coq complex (which is not over-expressed).

Discussion

In this study, we successfully rescued the yeast *coq9* temperature-sensitive mutant, *Δcoq9K*:TS19, with the human *COQ9* homolog expressed with a yeast mitochondrial leader sequence. Expression of human *COQ9* increased the growth of the *Δcoq9K*:TS19 mutant on respiratory media (Fig. 1) and Q content (Fig. 2) at both permissive and non-permissive temperatures. We also found that even though both multi-copy Coq8 and human COQ9 rescue TS19, human COQ9 dramatically increased the level of $^{13}C_6$-Q_6 when $^{13}C_6$-4HB was the precursor (Fig. 2C), but to a much lesser degree when $^{13}C_6$-pABA was the precursor (Fig. 2A). However, multi-copy Coq8 has a more significant effect on $^{13}C_6$-Q_6 productions when $^{13}C_6$-pABA was provided (Fig. 2A). These findings suggest that human Coq9 increases Q_6 production by promoting the conversion of 4HB to Q_6. Our findings are consistent with the observation that yeast can utilize pABA to synthesize Q (4), but not human cells (3). Yeast Coq9 controls the removal of nitrogen group of Q intermediates (12). It is possible that human Coq9 lacks this function so human cells do not use pABA as a Q precursor, but this hypothesis will require further investigation.

Human Coq9 failed to rescue yeast *coq9* null mutant, even with the over-expression of COQ8 to stabilize the rest of the CoQ-synthome (8) (Fig. 1). This might be due to the fact that yeast Coq9 is required for the function of yeast Coq6 and Coq7. Yeast *coq6* and *coq9* null mutants over-expressing *COQ8* both accumulate 4-AP when pABA is provided as the ring precursor and yeast *coq7* and *coq9* null mutants over-expressing *COQ8* both accumulate DMQ_6 when 4-HB is provided (10). Yeast Coq6 and Coq7 do not function well without yeast Coq9. Yeast Coq9 may play a structural or regulatory role to enable human Coq9 to function in yeast. Interestingly, the expression of human Coq9 stabilizes the steady state levels of Coq4, Coq6,

Coq7 and yeast Coq9 at permissive temperature. It was shown that supplementation of Q_6 to yeast mutants stabilizes CoQ-synthome and its Coq polypeptide subunits (8), so it is possible that human Coq9 stabilizes yeast Coq proteins by increasing Q_6 levels. Although we saw that Q_6 levels of Δ9K:TS19 were significantly increased by human Coq9 at both permissive and non-permissive temperature (Fig. 2), we only see human Coq9 stabilizes the steady state levels of yeast Coq polypeptides at permissive temperature (Fig. 3). This observation may be due to the fact that LC-MS/MS is highly sensitive and it can detect small changes in lipid levels, while Western blot may not be sensitive enough to show the changes in protein levels if the stabilizing effect of human Coq9 at non-permissive temperature is small.

In order to investigate the mechanism of human Coq9 rescuing yeast *coq9* mutant, we determine whether human Coq9 is associated with the CoQ-synthome. We performed consecutive non-denaturing affinity purification with CNAP tagged yeast Coq6. The structure of human COQ9 has recently identified it as a dimer (15). We speculated that human Coq9 might interact with yeast Coq polypeptides. We found that a small amount of human Coq9, along with yeast Coq9, co-precipitates with Coq6-CNAP. Therefore, human Coq9 interacts with the CoQ-synthome.

In conclusion, we found that human Coq9 could rescue the *coq9-ts19* mutant, possibly by interacting with the CoQ-synthome and stabilizing the complex by increasing Q_6 content derived from 4HB.

Table 1

Genotype and Source of Yeast Strains

Strain	Genotype	Source
W3031B	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303Δcoq4	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq4::TRP1*	(26)
W303Δcoq6	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq6::LEU2*	(27)
W303Δcoq7	MAT **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq7::LEU2*	(28)
BY4741Δcoq9	MAT a *coq9Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	(29)[b]
W303Δcoq9K	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 coq9::KanMX4*	This study
Coq6-CNAP	Mat **a** *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 COQ6::COQ6-CNAP-HIS3*	(6)

[a] Dr. Rodney Rothstein, Department of Human Genetics, Columbia University

[b] European S. cerevisiae Archive for Functional Analysis (EUROSCARF), available on-line

Table 2

Description and Source of Antibodies

Antibody	Working Dilution	Source
Atp2	1:4000	Carla M. Koehler[a]
Coq4	1:250	(30)
Coq6	1:250	(27)
Coq7	1:1000	(31)
Coq9	1:1000	(32)
Human Coq9	1:1000	Proteintech Group, Inc

[a] Dr. Carla M. Koehler, Department of Chemistry and Biochemistry, UCLA

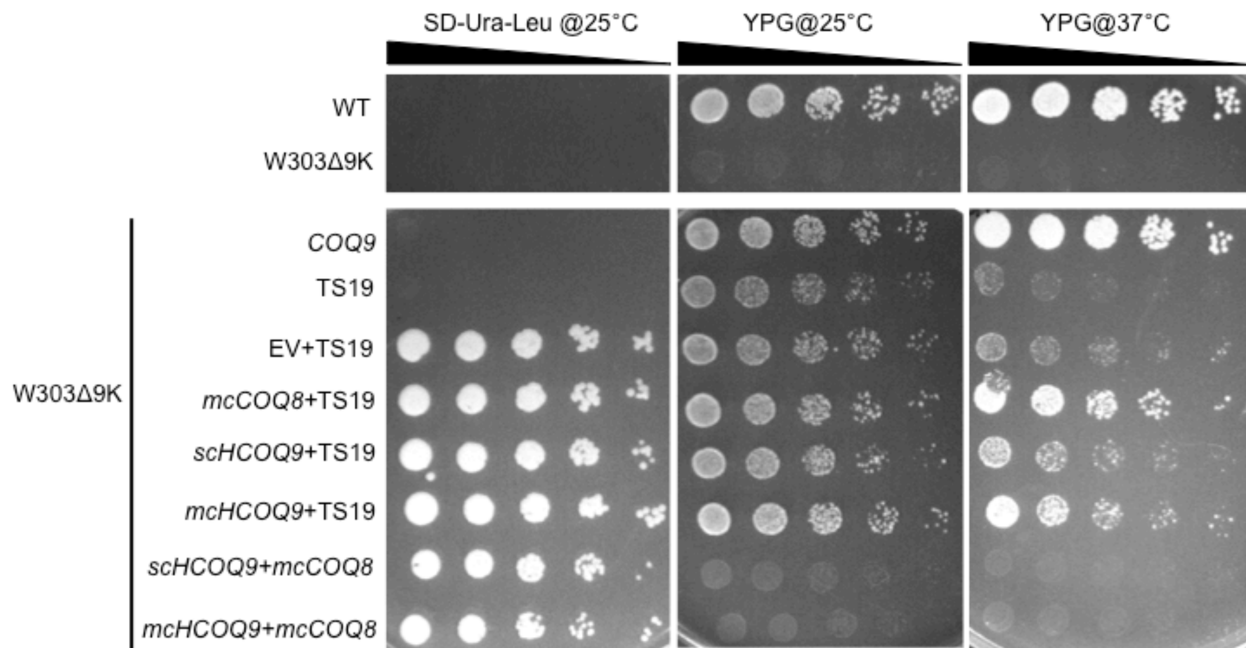
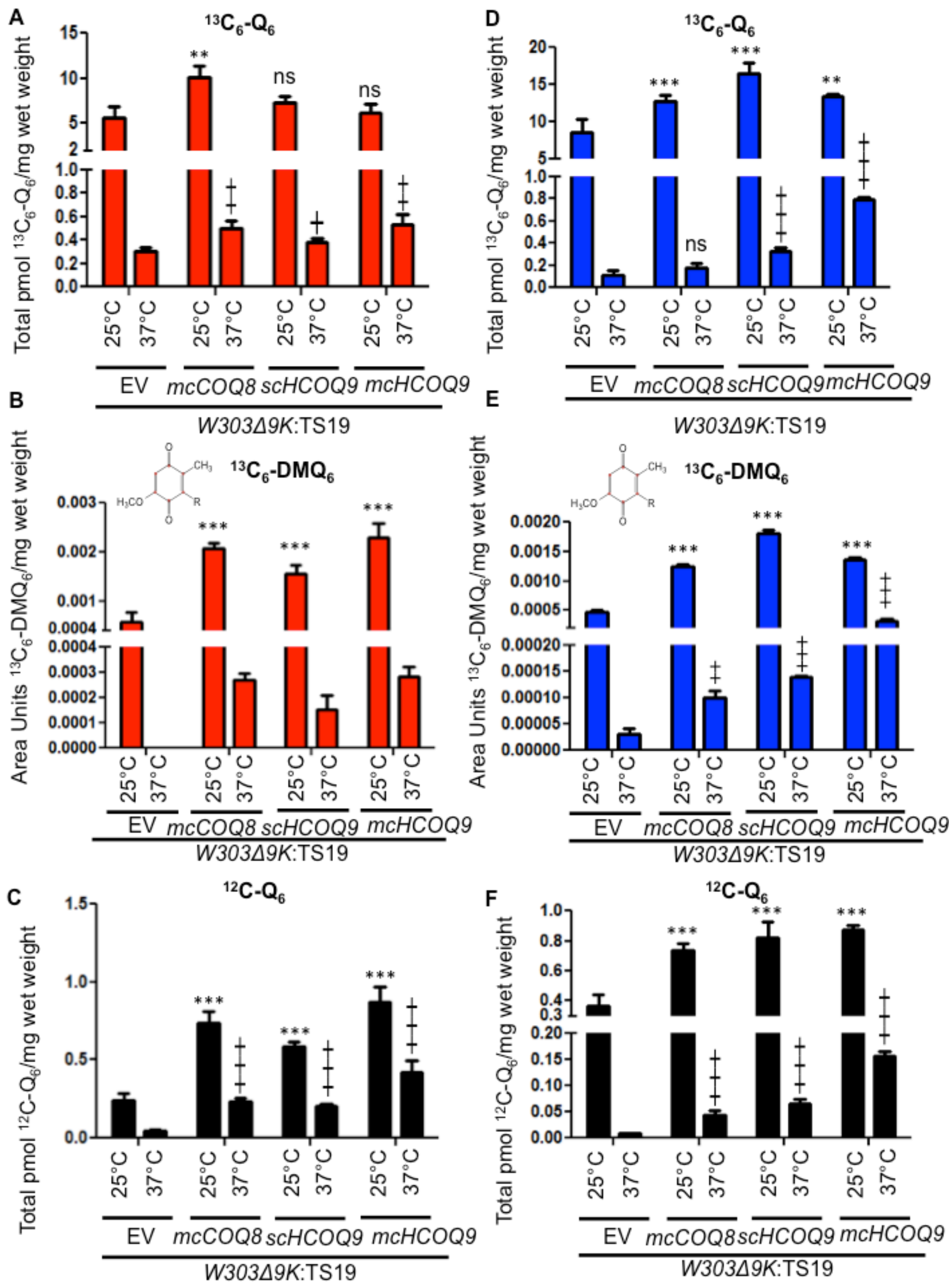


Fig. 1. Expression of human *COQ9* or over-expression of *COQ8* rescues the growth of the temperature-sensitive *coq9* mutant on a non-fermentable carbon source. W303Δ9K was transformed with TS19 and one of the following plasmids: empty vector pRS426 (EV), multi-copy yeast *COQ8* (*mcCOQ8*), single-copy human *COQ9* (*scHCOQ9*), and multi-copy human *COQ9* (*mcHCOQ9*). Yeast strains were cultured in SD−Leu−Ura media overnight at 25 °C. W3031B (WT), W303Δ9K, W303Δ9K:*COQ9*, and W303Δ9K:TS19 were used as controls and grown in YPG and SD−Leu respectively. Cell cultures were diluted to 0.2 A_{600nm} and 2 µl of 1:5 serial dilutions were spotted onto SD−Ura−Leu or YPG plate media and incubated at either 25°C or 37°C for 3 days.



A
$^{13}C_6$-Q_6
Total pmol $^{13}C_6$-Q_6/mg wet weight
15
10
5
2
1.0
0.8
0.6
0.4
0.2
0.0
**
ns
ns
25°C
37°C
EV
mcCOQ8
scHCOQ9
mcHCOQ9
B
$^{13}C_6$-DMQ_6
Area Units $^{13}C_6$-DMQ_6/mg wet weight
0.003
0.002
0.001
0.0004
0.0003
0.0002
0.0001
0.0000
***
W303Δ9K:TS19
C
^{12}C-Q_6
Total pmol ^{12}C-Q_6/mg wet weight
1.5
1.0
0.5
0.0
D
$^{13}C_6$-Q_6
20
15
10
5
ns
E
$^{13}C_6$-DMQ_6
0.0020
0.0015
0.0010
0.0005
0.00020
0.00015
0.00010
0.00005
0.00000
F
^{12}C-Q_6
0.3
0.20
0.15
0.10
0.05
0.00

Fig. 2. Expression of human *COQ9* or over-expression of *COQ8* increases the content of Q_6 and DMQ_6 in W303Δ9K expressing the temperature-sensitive plasmid TS19. W303Δ9K was transformed with TS19 and one of the following plasmids: empty vector pRS426, multi-copy of yeast *COQ8* (*mcCOQ8*), single-copy of human *COQ9* (*scHCOQ9*), and multi-copy of human *COQ9* (*mcHCOQ9*). One colony of each type of yeast transformant was seeded in selective media, SD−Ura−Leu, and grown overnight. The cell culture was diluted to $0.1A_{600nm}$ in 20 ml of fresh SD−Ura−Leu containing 10 μg/ml $^{13}C_6$-pABA or 10 μg/ml $^{13}C_6$-4HB dissolved in 2 μl ethanol/ml medium and grown at 25°C or 37°C for 12.5 hours. Final cell density was between 3 and 5 A_{600nm}. Yeast cells (corresponding to a total of 50 A_{600nm}) were collected as pellets, from which lipids were extracted and analyzed by RP-HPLC-MS/MS. Each bar represents a total four measurements from two independent samples each with two injections. Black bars represent the amount of ^{12}C-Q_6, red bars represent $^{13}C_6$-Q_6 and $^{13}C_6$-DMQ_6 labeled by $^{13}C_6$-pABA and blue bars represent $^{13}C_6$-Q_6 and $^{13}C_6$-DMQ_6 labeled by $^{13}C_6$-4HB. The amounts of the ^{12}C- and $^{13}C_6$-compounds represent the sum of reduced and oxidized forms. Both Q_6 and $^{13}C_6$-DMQ_6 levels were higher in W303Δ9K:TS19 harboring human *COQ9* homolog or over-expression of *COQ8* as compared to W303Δ9K:TS19 harboring empty vector as determined by the Student's two-tailed *t*-test. The *symbols represent samples at 25 °C compared to W303Δ9K:TS19+EV at 25 °C; *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$. The + symbols represent samples at 37 °C compared to W303Δ9K:TS19+EV at 37 °C; +, $p<0.05$, ‡, $p<0.01$, ‡, $p<0.001$. When there is no significant change, ns was used to designate "non-significant".

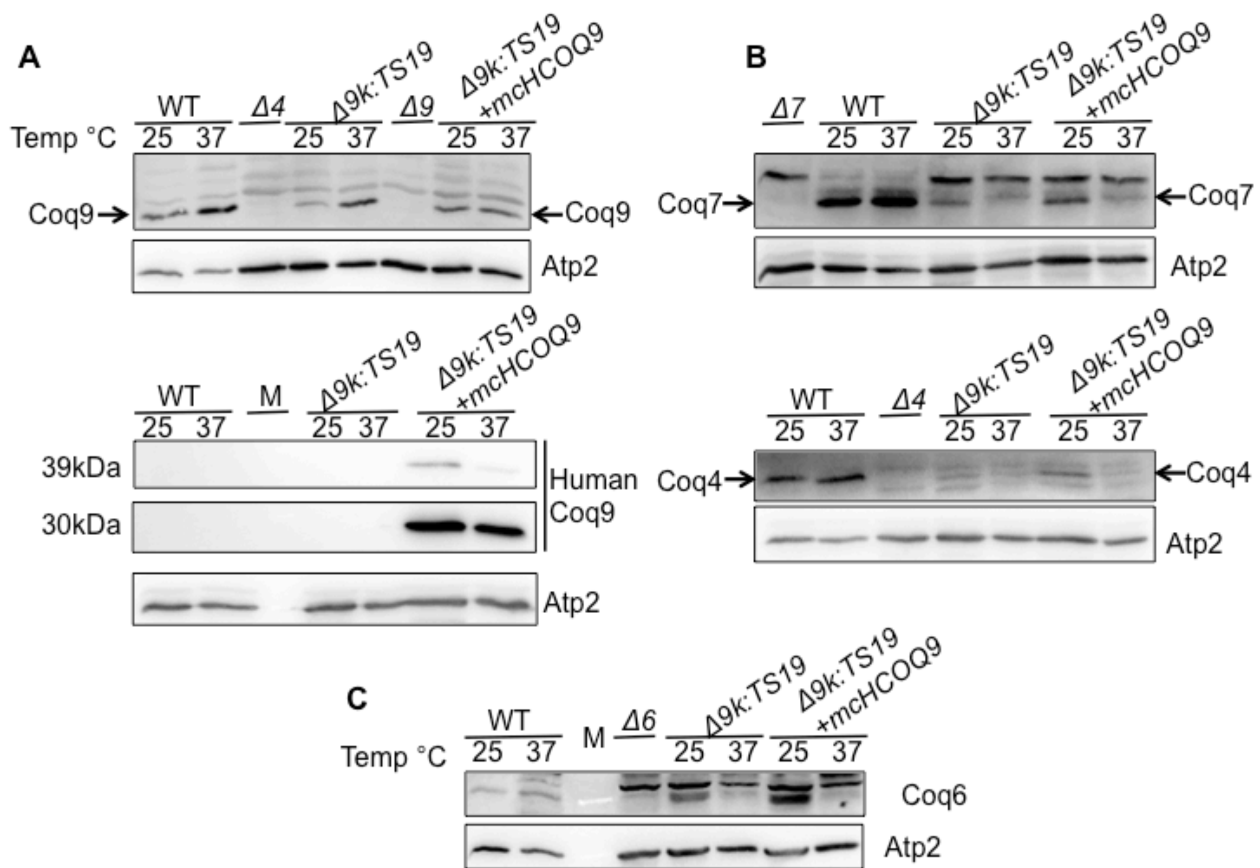


Fig. 3. Expression of human COQ9 stabilizes yeast Coq polypeptides in the temperature-sensitive *coq9* mutant at permissive temperature. W303Δ9K harboring the temperature-sensitive plasmid TS19 (Δ9K:TS19) were transformed with multi-copy human *COQ9* (Δ9K:TS19+*mcHCOQ9*). Yeast strains W3031B (WT), Δ9K:TS19, and Δ9K:TS19+*mcHCOQ9* were grown for 18.5 hours at either 25°C or 37°C. Mitochondria were then purified from these yeast cultures. Mitochondria were also isolated from the null control strains BY4741ΔCOQ9 (Δ9), W303ΔCOQ4 (Δ4), W303ΔCOQ7 (Δ7), and W303ΔCOQ6 (Δ6) after yeast were grown overnight at 30 °C. Purified mitochondria (15 μg protein) were separated by SDS-PAGE and analyzed by Western blot. Immunoblots were performed with antibodies against the designated polypeptides: Coq4, Coq6, Coq7, Coq9, human Coq9 and Atp2.

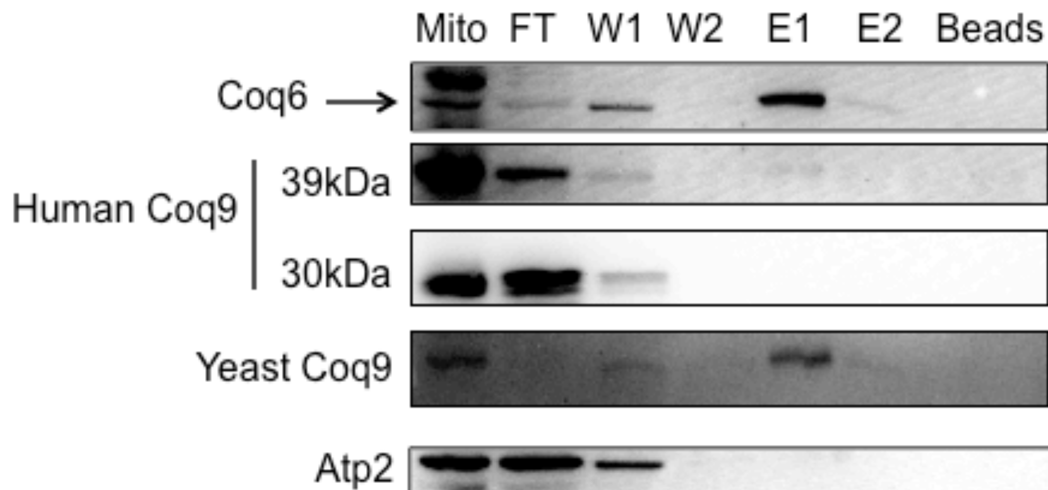


Fig. 4. The human Coq9 polypeptide associates with yeast Coq6. A consecutive non-denaturing tag containing a His_{10} tag and protein C epitope was integrated at the carboxyl end of Coq6, resulting in the strain CNAP6. CNAP6 was transformed with multi-copy human *COQ9* (CNAP6: *mcHCOQ9*). Mitochondria were isolated from CNAP6 and CNAP6: *mcHCOQ9*. Purified mitochondria (13 mg) were solubilized. Immunoprecipitation was then performed on the solubilized mitochondria with Ni-NTA resin. Flow-through (FT), wash (W1 and W2), eluate (E1 and E2), and beads from immunoprecipitation were collected. We analyzed 0.17% of the FT, 0.25% of W1, 0.25% of W2, 1% of E1, 0.5% of E2, and 1.25% of Ni-NTA resin by SDS-PAGE followed by immunoblotting with antibodies against yeast Coq9, Coq6, human Coq9 and Atp2. Purified mitochondria (15 µg) from CNAP6: *mcHCOQ9* were included as control.

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Chapter 5

Resveratrol and *para*-coumarate serve as ring precursors for coenzyme Q biosynthesis

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Resveratrol and *para*-coumarate serve as ring precursors for coenzyme Q biosynthesis[S]

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Abstract **Coenzyme Q (Q or ubiquinone) is a redox-active polyisoprenylated benzoquinone lipid essential for electron and proton transport in the mitochondrial respiratory chain. The aromatic ring 4-hydroxybenzoic acid (4HB) is commonly depicted as the sole aromatic ring precursor in Q biosynthesis despite the recent finding that *para*-aminobenzoic acid (pABA) also serves as a ring precursor in *Saccharomyces cerevisiae* Q biosynthesis. In this study, we employed aromatic $^{13}C_6$-ring-labeled compounds including $^{13}C_6$-4HB, $^{13}C_6$-pABA, $^{13}C_6$-resveratrol, and $^{13}C_6$-coumarate to investigate the role of these small molecules as aromatic ring precursors in Q biosynthesis in *Escherichia coli*, *S. cerevisiae*, and human and mouse cells. In contrast to *S. cerevisiae*, neither *E. coli* nor the mammalian cells tested were able to form $^{13}C_6$-Q when cultured in the presence of $^{13}C_6$-pABA. However, *E. coli* cells treated with $^{13}C_6$-pABA generated $^{13}C_6$-ring-labeled forms of 3-octaprenyl-4-aminobenzoic acid, 2-octaprenyl-aniline, and 3-octaprenyl-2-aminophenol, suggesting UbiA, UbiD, UbiX, and UbiI are capable of using pABA or pABA-derived intermediates as substrates. *E. coli*, *S. cerevisiae*, and human and mouse cells cultured in the presence of $^{13}C_6$-resveratrol or $^{13}C_6$-coumarate were able to synthesize $^{13}C_6$-Q. Future evaluation of the physiological and pharmacological responses to dietary polyphenols should consider their metabolism to Q.**—Xie, L.X., K. J. Williams, C. H. He, E. Weng, S. Khong, T. E. Rose, O. Kwon, S. J. Bensinger, B. N. Marbois, and C. F. Clarke. **Resveratrol and *para*-coumarate serve as ring precursors for coenzyme Q biosynthesis. *J. Lipid Res.* 2015. 56: 909–919.**

Supplementary key words antioxidants • isoprenoids • lipids/chemistry • mass spectrometry • mitochondria • ubiquinone • plant polyphenols • stilbene

Coenzyme Q (Q or ubiquinone) is a polyisoprenylated benzoquinone lipid essential for electron and proton transport in the mitochondrial respiratory chain and in the plasma membrane of *Escherichia coli* (1, 2). The hydroquinone or reduced form [coenzyme QH_2 or ubiquinol (QH_2)] functions as a chain-terminating lipid antioxidant and as a coantioxidant to recycle vitamin E (3). Q is also involved in many other metabolic processes, including fatty acid β-oxidation, sulfide oxidization, disulfide bond formation, and pyrimidine metabolism (4–7). Q is composed of a fully substituted benzoquinone ring that is attached to a polyisoprenyl tail with a variable number of isoprenyl units (six for *Saccharomyces cerevisiae*, eight for *E. coli*, nine for mouse, and ten for human, hence Q_{10}).

Most cells rely on de novo synthesis for sufficient amounts of Q, although brown adipose tissue was recently discovered to rely on uptake of exogenously supplied Q (8). In baker's yeast, *S. cerevisiae*, at least 13 gene products, Coq1-Coq11, Arh1, and Yah1 (9–14) are essential for Q biosynthesis. The Coq1 polypeptide synthesizes the polyisoprenyl tail and Coq2 attaches the tail to the aromatic ring (**Fig. 1**). The other Coq polypeptides catalyze ring modifications including *O*-methylation (Coq3), *C*-methylation (Coq5), hydroxylation (Coq6 and Coq7), and the function of Coq6 requires ferredoxin (Yah1) and ferredoxin reductase (Arh1) (9). The roles of Coq4, Coq8, Coq9, Coq10, and Coq11 have not yet been determined, although they are all required for efficient yeast Q biosynthesis. Schemes of Q biosynthesis generally depict 4-hydroxybenzoic acid (4HB) as the biosynthetic aromatic ring precursor of Q (4). 4HB is considered to derive from chorismate in yeast and from phenylalanine or tyrosine in

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Abbreviations: $[^{13}C_6]$pABA, *p*-amino[aromatic-$^{13}C_6$]benzoic acid; DoD, drop out dextrose; 4HB, 4-hydroxybenzoic acid; LB, Luria broth; OA, 2-octaprenyl-aniline; OAB, 3-octaprenyl-4-aminobenzoic acid; OAP, 2-amino-3-octaprenylphenol; OP, 2-octaprenyl phenol; pABA, *para*-aminobenzoic acid; p-coumarate, *para*-coumarate; Q, coenzyme Q or ubiquinone; QH_2, coenzyme QH_2 or ubiquinol; Q_n, coenzyme Q with n number of isoprene units in the polyisoprenoid tail; RP-HPLC-MS/MS, reverse phase-HPLC-MS/MS; TFA, trifluoroacetic acid.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of four figures.

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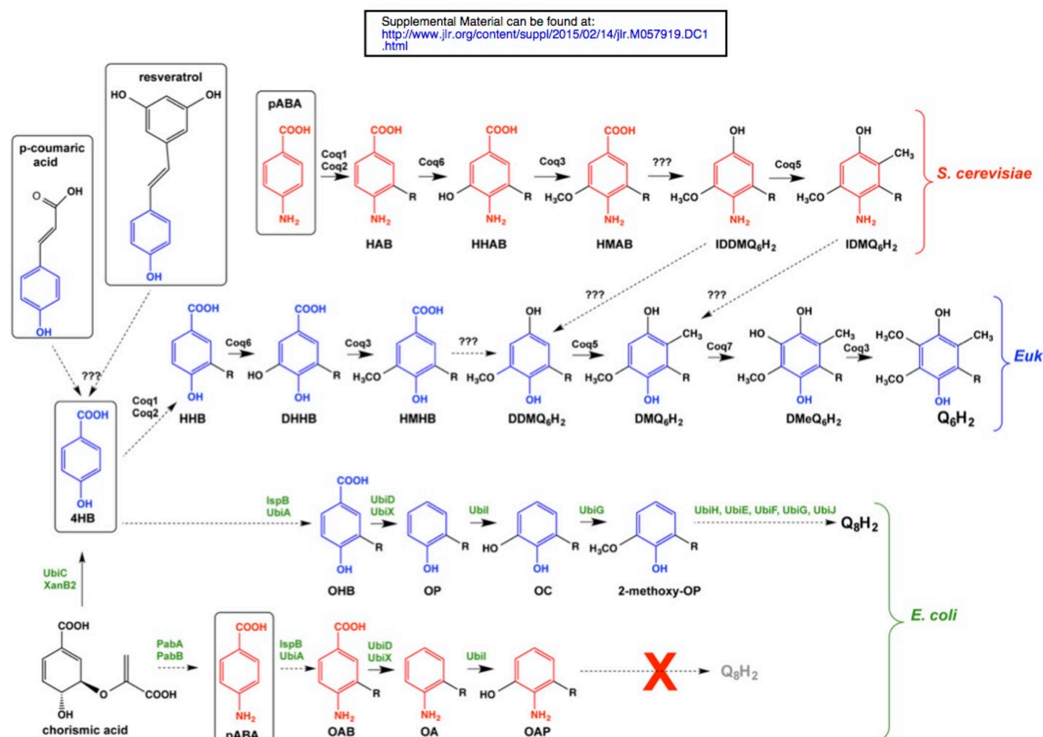

Fig. 1. Schemes of Q biosynthesis in *S. cerevisiae*, other eukaryotes, and *E. coli*. In *S. cerevisiae*, Coq1 synthesizes the hexaprenyl diphosphate tail, and Coq2 adds the hexaprenyl tail (denoted as "R") to either 4HB or to pABA, forming 3-hexaprenyl-4HB (HHB) or 3-hexaprenyl 4-aminobenzoic acid (HAB). Coq6 adds the first hydroxyl group to the C5 position of the aromatic ring, forming either 3-hexaprenyl-4,5-dihydroxybenzoic acid (DHHB) or 3-hexaprenyl-5-hydroxy-4-aminobenzoic acid (HHAB). An undetermined enzyme catalyzes the decarboxylation step, forming demethyl-demethoxy Q_6 ($DDMQ_6$) or imino-demethyl-demethoxy-Q_6 ($IDDMQ_6$). Coq5 catalyzes the *C*-methylation at the C2 position of the aromatic ring, producing either demethoxy-Q_6 (DMQ_6) or imino-demethoxy-Q_6 ($IDMQ_6$). The 4HB and pABA branches are proposed to converge at the steps designated by the dotted arrows. Coq7 adds the second OH group to the C6 position, generating demethyl-Q_6 ($DMeQ_6$), followed by the second *O*-methylation catalyzed by Coq3 to synthesize Q_6. Coq4, Coq9, Coq10, and Coq11 are required for efficient Q_6 biosynthesis, but their function is yet to be determined. Human and mouse cells (depicted as "Euk") produce Q_{10} and Q_9 via steps similar to those shown for *S. cerevisiae*. *E. coli* proteins responsible for Q_8 biosynthesis are designated with green text. UbiC converts chorismate to 4HB. IspB synthesizes the octaprenyl diphosphate tail and UbiA adds the octaprenyl tail (denoted as "R") to the 4HB or pABA to form 3-octaprenyl-4HB (OHB) or OAB. UbiD and/or UbiX catalyze the decarboxylation of the aromatic ring forming OP or OA. UbiI adds the first hydroxyl to the ring to form octaprenylcatechol (OC) or 2-amino-3-octaprenylphenol (OAP). The pABA branch of the pathway stops at this step, while UbiG *O*-methylates OC to form 2-methoxy-OP. Additional ring modifications catalyzed by UbiH, UbiE, UbiF, and UbiG form the final product Q_8H_2. UbiB and UbiJ are required for Q_8 biosynthesis, but their function is yet to be determined. Boxed compounds designate the aromatic ring precursors tested in this study.

animal cells (15–17). Yeast can also use *para*-aminobenzoic acid (pABA) as an alternate ring precursor in the biosynthesis of Q (9, 18). This finding was surprising because pABA is a well-known precursor of folate, which is synthesized de novo by many microorganisms and folate is a vitamin for humans. A biosynthetic scheme was reported recently including proposed steps for the conversion of pABA to Q_6 in *S. cerevisiae* (19).

The biosynthesis of Q_8 in *E. coli* requires IspB (which synthesizes the octaprenyl diphosphate tail precursor) (20) and 11 Ubi polypeptides (UbiA–UbiJ and UbiX; Fig. 1) (21). UbiC carries out the first committed step in the biosynthesis of Q_8, the conversion of chorismate to 4HB (22). UbiA adds the octaprenyl tail to the 4HB ring, followed by the decarboxylation catalyzed by UbiD and UbiX. UbiI adds the first hydroxyl group at the C5 position, followed by *O*-methylation catalyzed by UbiG, the homolog of yeast Coq3. Additional ring modifications catalyzed by UbiH, UbiE, UbiF, and UbiG generate the final product of Q_8. UbiB, an atypical protein kinase similar

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to Coq8, and UbiJ play essential, but unknown, functions in *E. coli* Q_8 biosynthesis (21).

Recently, Block et al. (15) identified *para*-coumarate (p-coumarate) as a ring precursor of Q biosynthesis in *Arabidopsis thaliana. Arabidopsis* converts phenylalanine to p-coumarate in the cytosol, and following transport into peroxisome, p-coumarate is ligated to CoA and the three-carbon side chain is shortened via peroxisomal β-oxidation (15). Plant peroxisomes appear to contain thiolases and CoA thioesterases that can ultimately produce 4HB from 4-hydroxybenzoyl-CoA (15). Tyrosine can also supply the ring of Q in *Arabidopsis*; but this must occur via a nonintersecting pathway, because *Arabidopsis* mutants unable to utilize phenylalanine still utilized tyrosine as a ring precursor of Q (15). Animal cells are able to hydroxylate phenylalanine to form tyrosine, and it is presumed that conversion of tyrosine to 4HB occurs via its metabolism to p-coumarate (16, 23). However, the enzymes involved in 4HB biosynthesis in either yeast or animal cells have not been identified.

The in vivo metabolism of potential ring precursors labeled with the stable isotope ^{13}C can be determined with high sensitivity and specificity with reverse phase (RP)-HPLC-MS/MS identification and quantification. Using this approach, Block et al. (15) showed that *Arabidopsis* was not able to incorporate $^{13}C_6$-pABA into Q. Here, we have made use of $^{13}C_6$-ring-labeled forms of pABA and p-coumarate to track their metabolic fate as potential Q biosynthetic precursors in *E. coli, S. cerevisiae,* and animal cells. Due to its structural similarity with p-coumarate, $^{13}C_6$-resveratrol was also tested as a ring precursor in Q biosynthesis. In this study, we found that human and *E. coli* cells do not utilize pABA as an aromatic ring precursor in the synthesis of Q, while resveratrol and p-coumarate serve as ring precursors of Q in *E. coli, S. cerevisiae,* and human cells.

MATERIALS AND METHODS

Yeast growth and stable isotope labeling

The *S. cerevisiae* strains used are described in **Table 1**. YPD medium (2% glucose, 1% yeast extract, 2% peptone) was prepared as described (24). Solid plate medium included the stated components plus 2% Bacto agar. Yeast colonies from YPD plate medium were first inoculated into 250 ml flasks containing 70 ml YPD liquid medium. Following overnight incubation with shaking (250 rpm) at 30°C, yeast cells were transferred into fresh drop out dextrose (DoD) medium (18). DoD medium contained 2% dextrose, 6.8 g/l Bio101 yeast nitrogen base minus pABA minus folate with ammonium sulfate (MP Biomedicals), and 5.83 mM sodium monophosphate (pH adjusted to 6.0 with NaOH). Amino acids and nucleotides were added as described previously (18).

Stable isotope-containing compounds included *p*-hydroxy-[aromatic-$^{13}C_6$]benzoic acid ([$^{13}C_6$]4HB) from Cambridge Isotope Laboratories (Andover, MA); resveratrol[4-hydroxyphenyl-$^{13}C_6$] ([$^{13}C_6$]resveratrol), and *p*-amino[aromatic-$^{13}C_6$]benzoic acid ([$^{13}C_6$]pABA) from Sigma/ISOTEC (Miamisburg, OH). During this work, we discovered that preparations of [$^{13}C_6$]pABA supplied by Cambridge Isotope Laboratories were contaminated with approximately 1% [$^{13}C_6$]4HB. This small level of contamination confounded the initial labeling studies we performed. All studies reported here were performed with the [$^{13}C_6$]pABA obtained from Sigma/ISOTEC, and there was no detectable contamination with [$^{13}C_6$]4HB present in either the [$^{13}C_6$]pABA or [$^{13}C_6$]resveratrol (data not shown).

$^{13}C_6$-labeled aromatic ring precursors were added to fresh DoD medium and incubated with yeast cells (100 A_{600}) at 30°C for 4 h. Cells were collected by centrifugation and pellets were stored at −20°C. The wet weight of each cell pellet was determined by subtracting the weight of the tube from the total weight. Protein assays (BCA assay, Thermo) were performed on yeast cell lysates (25). For $^{13}C_6$-coumarate labeling, BY4741 yeast cells were incubated in 5 ml of SD-complete medium at a starting cell density of 0.1 A_{600} and incubated at 30°C for 24 h. The yeast cell density after incubation was approximately 6 A_{600}.

Synthesis of p-coumaric acid [aromatic-$^{13}C_6$]

The synthesis was similar to the method described by Robbins and Schmidt (26), with the following modifications. To a flame-dried flask (25 ml) was added 4-hydroxybenzaldehyde [aromatic-$^{13}C_6$] (50 mg), malonic acid (75 mg), piperidine (5 μl), and pyridine (1 ml). The reaction mixture was stirred under argon at 92°C. The reaction was monitored through thin layer chromatography on 0.25 mm SiliCycle silica gel plates and visualized under UV light and with permanganate or 2,4-dinitrophenylhydrazine staining. Upon completion (12 h), the mixture was sequentially added to 10 ml water, neutralized to pH 7–8, and then washed with dichloromethane. The aqueous solution was acidified to pH 1 and then extracted twice using ethyl acetate. The combined organic extract was concentrated in vacuo and purified through flash column chromatography. Flash column chromatography was performed with SiliCycle Silica-P Flash silica gel (60 Å pore size, 40–63 μm) and 50% ethyl acetate in hexanes as mobile phase, to furnish an off-white solid (58 mg, 87% yield). A portion was further purified by semi-preparative RP-HPLC (Waters Sunfire C18,

TABLE 1. Genotype and source of *S. cerevisiae* and *E. coli* strains

Strain	Genotype	Source
S. cerevisiae		
W3031B	MAT α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
BY4741	MAT a *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
E. coli		
HW272	ubiG+ *zei*::Tn*10*dTet	(52)
BW25113	*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1*	(27)
BW25113*ubiC*	*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1 ΔubiC::kan*	(27)
MG1655	F$^-$ lambda$^-$ *ilvG$^-$ rfb-50 rph-1*	(53)
MG1655*ubiC*	MG1655+P1/JW5713 (10), selection LB kan	F. Pierrel[b]

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[b]Dr. Fabien Pierrel, Laboratoire de Chimie et Biologie des Métaux, Université Grenoble.

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10×250 mm, 5 μm) using the following gradient elution (solvent A: water + 0.1% trifluoroacetic acid (TFA), solvent B: acetonitrile + 0.1% TFA, flow rate 6.0 ml/min): 0–2 min 10% B; 2–20 min linear 10→45% B; 20–22 min linear 45–10% B; 22–25 min 10% B. Fractions were pooled, concentrated in vacuo, and the aqueous remainder was lyophilized to give a white powder (11.8 mg). RP-HPLC analysis indicated >99% purity at 210 and 254 nm, and no detectable 4HB (Waters Sunfire C18, 4.6×250 mm, 5 μM; solvents A/B as above, flow rate 1.00 ml/min) using the following gradient elution: 0–1 min 10% B; 1–20 min linear 10–100% B; 20–25 min 100% B; 25–27 min linear 100–10% B; 27–30 min 10% B.

NMR spectra were recorded using a Bruker Avance-500 spectrometer, calibrated to residual acetone-d_6 as the internal reference (2.05 ppm for ^{1}H NMR; 29.9 and 206.7 ppm for ^{13}C NMR). ^{1}H NMR spectral data are reported in terms of chemical shift (δ, parts per million), multiplicity, coupling constant (hertz), and integration. ^{13}C NMR spectral data are reported in terms of chemical shift (δ, parts per million), multiplicity, and coupling constant (hertz). The following abbreviations indicate the multiplicities: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. ^{1}H NMR (500 MHz, acetone-d_6) δ 9.40 (br s, 1H), 7.70–7.65 (m, 1H), 7.63–7.57 (m, 1H), 7.39–7.34 (m, 1H), 7.05–7.02 (m, 1H), 6.71–6.69 (m, 1H), 6.32 (dd, J = 5.2, 16.1 Hz, 1H) (supplementary Fig. 1A); ^{13}C NMR (125 MHz, acetone-d_6) δ 159.5 (dt, J = 64.8, 8.6 Hz), 129.9 (tt, J = 58.8, 4.4 Hz), 125.9 (dt, J = 58.0, 9.2 Hz), 115.6 (dt, J = 64.6, 4.1 Hz) (supplementary Fig. 1B). GC-MS data were recorded using an Agilent 6890-5975 GC mass spectrometer equipped with an autosampler and an HP5 column; the sample was dissolved in ethanol. GC-MS (EI+) calculated for $^{13}C_6{}^{12}C_3H_8O_3$ M$^+$, m/z 170.1, found 170.1.

E. coli growth and stable isotope labeling

E. coli strains are described in Table 1. The BW25113 Δ*ubiC::kan* mutant strain was obtained from the Keio collection (27). Phage P1 was used to transduce the mutation into the MG1655 strain, yielding MG1655*ubiC*. The replacement of the chromosomal *ubiC* gene by the *kan* gene was checked by PCR amplification. Cells were inoculated in 100 ml of Luria broth (LB) for 16 h at 37°C. Cells (50 A_{600}) from each sample were collected by centrifugation, and the collected pellets were resuspended in fresh LB medium in the presence of either 10 μg/ml of $^{13}C_6$-4HB, $^{13}C_6$-pABA, or $^{13}C_6$-resveratrol, and incubated at 37°C with shaking at 250 rpm. Incubations with vehicle control contained an equivalent volume of ethanol (in all conditions the final ethanol concentration was 0.2%). Cells were collected by centrifugation after 4 h and stored at −20°C for LC-MS/MS lipid analyses. For $^{13}C_6$-coumarate labeling, HW272, HW25113, MG1655, and MG1655ubiC were inoculated in 5 ml of LB for 16 h at 37°C (MG1655ubiC was incubated in LB with 50 μg/ml kanamycin). Cells were diluted to 0.2 A_{600} in fresh media with 15 μg/ml of $^{13}C_6$-coumarate and incubated for 24 h. Cells were pelleted for lipid extraction and LC-MS/MS analyses.

Animal cell culture and stable isotope labeling

U251 human glioma and 3T3 mouse fibroblast cells were cultured in DMEM (Gibco). U87 human glioma cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco). Human embryonic kidney 293T cells were cultured in DMEM with 1 mM sodium pyruvate (Gibco). All cells were passaged in the stated media supplemented with 10% FBS (Omega Scientific) and 1% penicillin-streptomycin (10,000 U/ml) (Life Technologies). Equal numbers of cells were plated approximately 12 h prior to treatment experiments. During treatment with stable isotope-labeled compounds, cells were cultured with 1% FBS, unless otherwise stated. Cells were cultured with the designated stable isotope-labeled compound for 24 h, then washed with PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH 7.4)], and released from the culture dish with 0.25% trypsin-EDTA (Gibco). Aliquots of the released cells were stained with Trypan blue and the number of cells counted with the Cellometer Auto T4 (Nexcelom Bioscience); aliquots (5%) were also removed for determination of protein content (BCA assay; Thermo). The remaining cells in the suspension were collected by centrifugation. Cell pellets were stored at −20°C.

Lipid extraction

Cell pellets were thawed on ice and then suspended in 1.2 ml of methanol followed by 1.8 ml of petroleum ether. Q_4 was added as an internal standard for the determination of Q_6 content in

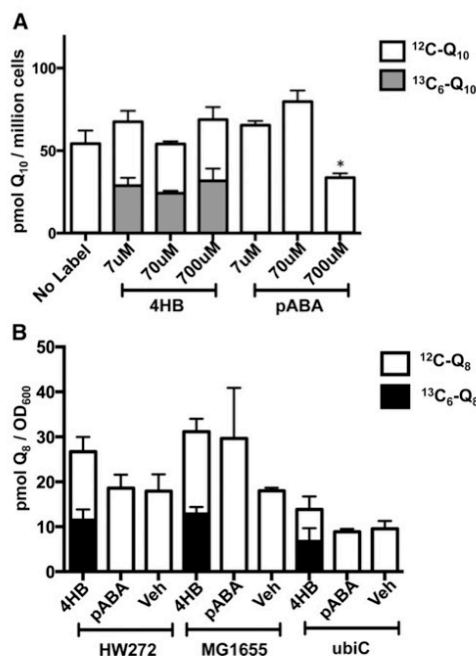

Fig. 2. pABA is not utilized as an aromatic ring precursor to Q in mammalian or *E. coli* cells. A: Human glioblastoma (U251) cells were cultured and processed as described in the Materials and Methods. The plots show the total Q_{10} content detected by RP-HPLC-MS/MS under designated precursor conditions. The gray bar of each column represents $^{13}C_6$-Q_{10}, while the white bar represents ^{12}C-Q_{10}. Error bars represent standard deviation with n = 4. Cells were treated with the designated concentrations of either $^{13}C_6$-4HB or $^{13}C_6$-pABA. U251 cells incubated with 700 μM of $^{13}C_6$-pABA had a significantly lower amount of total Q (*$P < 0.05$, one-way ANOVA). B: HW272, MG1655, and MG1655*ubiC E. coli* cells were cultured and processed as described in the Materials and Methods section. The plots show total Q_8 content detected by RP-HPLC-MS/MS under designated conditions. The black bar of each column represents $^{13}C_6$-Q_8, while the white bar represents ^{12}C-Q_8. Error bars represent SD with n = 4. Only *E. coli* cells treated with $^{13}C_6$-4HB had detectable $^{13}C_6$-Q_8. Ethanol was used as vehicle control (Veh).

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yeast lipid extracts. Diethoxy-Q_{10} (28) was used as an internal standard for determination of Q_9 and Q_{10} in mammalian cell lipid extracts and Q_8 in *E. coli* cell lipid extracts. Samples were vortexed for 45 s, then the upper layer was removed to a new tube, and another 1.8 ml of petroleum ether was added to the lower phase and the sample was vortexed again for 45 s. The upper layer was again removed and combined with the previous organic phase. The combined organic phase was dried under a stream of nitrogen gas and resuspended in 200 μl of ethanol (USP; Aaper Alcohol and Chemical Co., Shelbyville, KY).

RP-HPLC-MS/MS

The RP-HPLC-MS/MS analyses were performed as previously described for determination of Q_6 in yeast lipid extracts (11, 18) and determination of Q_9 and Q_{10} in mammalian lipid extracts (28, 29). Briefly, a 4000 QTRAP linear MS/MS spectrometer from Applied Biosystems (Foster City, CA) was used. Applied Biosystem software, Analyst version 1.4.2, was used for data acquisition and processing. A binary HPLC solvent delivery system was used with either a Luna phenyl-hexyl column (particle size 5 μm, 100 × 4.60 mm; Phenomenex) for yeast cell lipid extracts or a Luna phenyl-hexyl column (particle size 3 μm, 50 × 2.00 mm; Phenomenex) for mammalian and bacteria cell lipid extracts. The mobile phase consisted of solvent A (methanol:isopropanol, 95:5, with 2.5 mM ammonium formate) and solvent B (isopropanol, 2.5 mM ammonium formate). For separation of yeast quinones, the percentage of solvent B increased linearly from 0 to 5% over 6 min, and the flow rate increased from 600 to 800 μl/min. The flow rate and mobile phase were linearly changed back to initial condition by 7 min. For separation of bacteria and mammalian quinones, the percentage of solvent B for the first 1.5 min was 0%, and increased linearly to 10% by 2 min. The percentage of solvent B remained unchanged for the next min and decreased linearly back to 0% by 6 min. A constant flow rate of 800 μl/min was used. All samples were analyzed in multiple reaction monitoring mode; multiple reaction monitoring transitions were as follows: *m/z* 591/197.1 (Q_6); *m/z* 610/197.1 (Q_6H_2 with ammonium adduct); *m/z* 597/203.1 ($^{13}C_6$-Q_6); *m/z* 616/203.1 ($^{13}C_6$-Q_6H_2 with ammonium adduct); *m/z* 636/106 [2-octaprenyl-aniline (OA)]; *m/z* 637/107 [2-octaprenyl phenol (OP)]; *m/z* 642/112 ($^{13}C_6$-OA); *m/z* 643/113 ($^{13}C_6$-OP); *m/z* 652/122 [2-amino-3-octaprenylphenol (OAP)]; *m/z* 658/128 ($^{13}C_6$-OAP); *m/z* 682/150

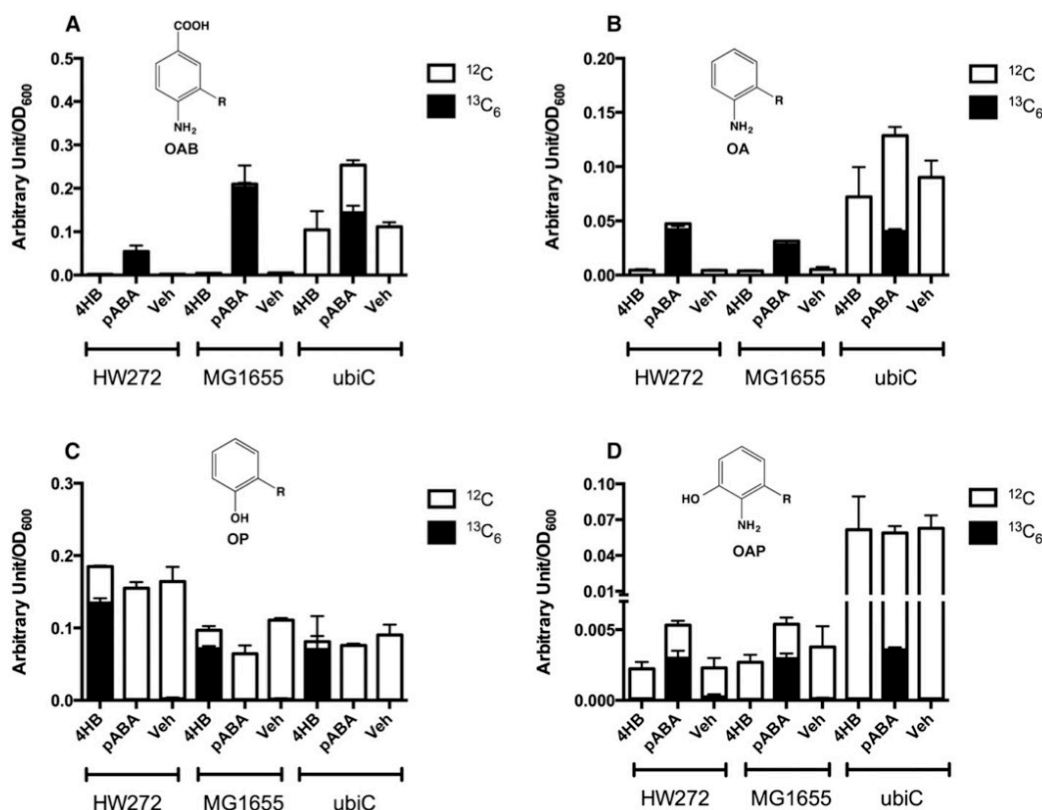

Fig. 3. *E. coli* cells produce $^{13}C_6$-pABA-derived octaprenyl-products. HW272, MG1655, and MG1655*ubiC* cells were cultured and processed as described in the Materials and Methods. Bar plots show the total content of OAB (A), OA (B), OP (C), and OAP (D). Each bar represents mean ± SD. The black bar of each column represents the designated $^{13}C_6$-labeled intermediate, while the white bar represents the ^{12}C-intermediate. Each *y* axis represents the area under the peak of interest first normalized by the internal standard (diethoxy-Q_{10}), and then by the value of OD_{600} of the extracted cell pellets.

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(OAB); *m/z* 688/156 ($^{13}C_6$-OAB); *m/z* 727/197.1 (Q_8); *m/z* 733/203.1 ($^{13}C_6$-Q_8); *m/z* 746/197.1 (Q_8H_2 with ammonium adduct); *m/z* 750/203.1 ($^{13}C_6$-Q_8H_2 with ammonium adduct); *m/z* 880.7/197.0 (Q_{10} with ammonium adduct); *m/z* 882.7/197.0 ($Q_{10}H_2$ with ammonium adduct); *m/z* 886.7/203.0 ($^{13}C_6$-Q_{10} with ammonium adduct); *m/z* 888.7/203 ($^{13}C_6$-$Q_{10}H_2$ with ammonium adduct); *m/z* 812.6/197 (Q_9 with ammonium adduct); *m/z* 455.6/197.1 (Q_4); *m/z* 908.7/225.1 (diethoxy-Q_{10} with ammonium adduct); and *m/z* 910.7/225.1 (diethoxy-$Q_{10}H_2$ with ammonium adduct).

RESULTS

pABA is a demonstrated ring precursor of Q biosynthesis in the yeast *S. cerevisiae* (9, 18), but is not utilized as a ring precursor of Q biosynthesis in *Arabidopsis* (15). To investigate whether pABA may serve as a ring precursor of Q biosynthesis in mammalian cells, human U251 cells were cultured in the presence of 7, 70, or 700 μM of either $^{13}C_6$-4HB or $^{13}C_6$-pABA for 24 h prior to RP-HPLC-MS/MS analysis of Q content (**Fig. 2A**). U251 cells readily converted $^{13}C_6$-4HB to $^{13}C_6$-Q_{10}, however, incubations with $^{13}C_6$-pABA produced no detectable $^{13}C_6$-Q_{10} (Fig. 2A, supplementary Fig. 2). Treatments of U251 cells with various $^{13}C_6$-4HB concentrations did not alter the total Q_{10} content; however incubation with 700 μM $^{13}C_6$-pABA resulted in significantly lower total Q_{10} content in mammalian cells ($P < 0.05$).

To examine whether pABA is utilized as a ring precursor in *E. coli* Q_8 biosynthesis, $^{13}C_6$-pABA or $^{13}C_6$-4HB was added to cultures of the designated *E. coli* strains. HW272 and MG1655 are wild-type strains, while MG1655*ubiC* contains a deletion of the *ubiC* gene encoding chorismate pyruvate lyase (Table 1). Each *E. coli* strain was cultured in LB medium with aromatic ring precursors added to a final concentration of 10 μg/ml (Fig. 2B). Each of the *E. coli* strains incubated in the presence of 10 μg/ml $^{13}C_6$-4HB accumulated significant amounts of $^{13}C_6$-Q_8. No incorporation of $^{13}C_6$-pABA into $^{13}C_6$-Q_8 was detected with the wild-type strains. Interestingly, the *E. coli ubiC* mutant was also unable to use pABA to synthesize Q_8. This result suggests that pABA is not utilized, even under conditions of impaired 4HB synthesis.

Detection of various polyprenylated derivatives of $^{13}C_6$-pABA indicated that the *E. coli* strains tested were able to take up this ring. For example, $^{13}C_6$-3-octaprenyl-4-aminobenzoic acid (OAB) indicated that $^{13}C_6$-pABA-treated *E. coli* cells successfully absorbed $^{13}C_6$-pABA from the medium and performed the ring prenyltransferase step catalyzed by UbiA (30) (**Fig. 3A**). $^{13}C_6$-OA (Fig. 3B) was also readily detected in lipid extracts of the $^{13}C_6$-pABA-treated *E. coli* cells. Notably, the *ubiC* mutant accumulated significantly more aniline-containing intermediates (Fig. 3A, B), even in the absence of pABA addition, presumably due to a deficiency in 4HB synthesis. The product $^{13}C_6$-OAP (Fig. 3D) was also observed in $^{13}C_6$-pABA-treated *E. coli* cells and is probably due to UbiI, which catalyzes the first hydroxylation step in Q_8 biosynthesis (31). The *ubiC* mutant accumulated 10 times more ^{12}C-OAP than HW272 or MG1655, a finding

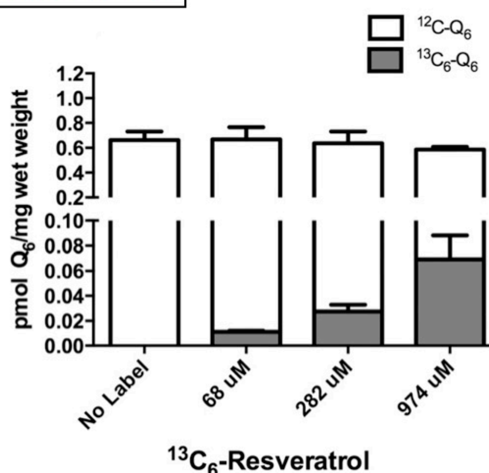

Fig. 4. $^{13}C_6$-resveratrol is a ring precursor to Q_6 biosynthesis in *S. cerevisiae*. Yeast W3031B wild-type cells incubated with 0, 68, 282, or 974 μM of $^{13}C_6$-resveratrol were cultured and analyzed as described in the Materials and Methods. The gray bar represents $^{13}C_6$-Q_6 and the white bar indicates ^{12}C-Q_6. Q_4 was used as the internal standard.

independent of the supplied $^{13}C_6$-pABA, suggesting that OAP might be a "dead-end" product. $^{13}C_6$-OAB, $^{13}C_6$-OA, or $^{13}C_6$-OAP were not detected in either the $^{13}C_6$-4HB-treated or control *E. coli* cells (Fig. 3A, B, D). $^{13}C_6$-OP was detected only in $^{13}C_6$-4HB-treated cells (Fig. 3C). These results suggest that although pABA is prenylated and can be further modified by UbiD, UbiX, and UbiI, *E. coli* may not be able to process the aniline-containing ring intermediates to later intermediates or to Q_8.

Given that *S. cerevisiae* can utilize either 4HB or pABA in Q_6 biosynthesis, we investigated the use of other possible aromatic ring precursors. Surprisingly, wild-type yeast could use resveratrol as a ring precursor in the synthesis of Q_6 (**Fig. 4**). W303 cells cultured in the presence of 68, 282, or 974 μM of $^{13}C_6$-resveratrol showed increasing amounts of $^{13}C_6$-Q_6, while the ethanol control samples contained no detectable $^{13}C_6$-Q_6. Notably, the increased amount of resveratrol did not alter the total Q_6 content. We next examined whether human or mouse cells could use resveratrol as a ring precursor to Q. The three human cell lines we examined were able to convert resveratrol to Q, as shown by the accumulation of $^{13}C_6$-Q_{10} (**Fig. 5A–C**, supplementary Fig. 3A–C). $^{13}C_6$-Q_9 (Fig. 5D, supplementary Fig. 3D) also accumulated in mouse 3T3 fibroblasts, when cultured in the presence of 70 μM of $^{13}C_6$-resveratrol. Although cells cultured with $^{13}C_6$-4HB accumulated significantly more $^{13}C_6$-Q_{10} than when cultured with $^{13}C_6$-resveratrol, the incorporation of $^{13}C_6$-resveratrol into $^{13}C_6$-Q_{10} accounted for approximately 10% of the total Q_{10}, a proportion that was much higher than that observed in wild-type yeast cells (the $^{13}C_6$-Q_6 was less than 1% of the total Q_6). $^{13}C_6$-Q_{10} content in U251 and $^{13}C_6$-Q_9 3T3 cells increased in response to the

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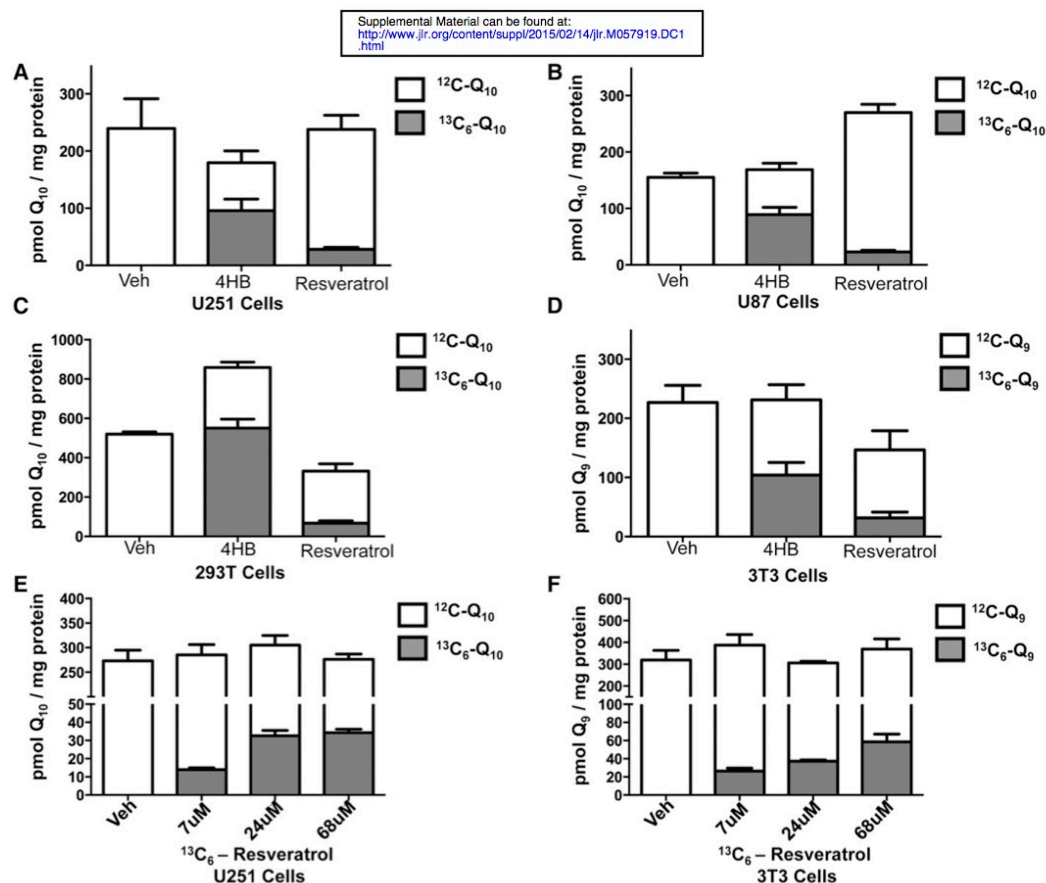

Fig. 5. Human and mouse cells utilize $^{13}C_6$-resveratrol as a ring precursor in Q biosynthesis. U251 cells (A); U87 cells (B); 293T cells (C); and 3T3 cells (D) were cultured in medium with 1.0% FBS in the presence of either $^{13}C_6$-4HB (278 μM), $^{13}C_6$-resveratrol (70 μM), or ethanol as vehicle control for 24 h prior to collection. Increasing concentrations of resveratrol does not alter total Q levels in mammalian cells: U251 (E) and 3T3 (F) cells cultured in the presence of 0, 7, 24, or 68 μM $^{13}C_6$-resveratrol were processed and analyzed. Collected cells were extracted and analyzed by RP-HPLC-MS/MS as described in the Materials and Methods. The gray bars represent $^{13}C_6$-containing Q, and the white bars represent ^{12}C-Q. Error bars represent SD (n = 4). Diethoxy Q_{10} was used as an internal standard.

increasing concentrations of $^{13}C_6$-resveratrol, while the total Q content again remained unaltered (Fig. 5E, F). Unfortunately, higher concentration (>70 μM) of resveratrol induced cell death, thus we were not able to examine the amount of $^{13}C_6$-Q synthesized in the presence of higher $^{13}C_6$-resveratrol concentrations.

E. coli also utilized resveratrol as an alternative ring precursor to Q, although to a lesser extent when compared with yeast, mouse, or human cells. HW272 and MG1655 cells cultured in LB medium in the presence of 10 μg/ml $^{13}C_6$-resveratrol accumulated trace amounts of $^{13}C_6$-Q_8 (**Fig. 6A**, supplementary Fig. 4A). In comparison, 10 μg/ml of $^{13}C_6$-4HB resulted in $^{13}C_6$-labeling of more than two-thirds of the total Q content in the same cells. However, the *E. coli ubiC* mutant, with a defect in de novo synthesis of 4HB, produced significantly more $^{13}C_6$-Q_8 when treated with $^{13}C_6$-resveratrol (Fig. 6A, supplementary Fig. 4B). $^{13}C_6$-OP was detected only when *E. coli* strains were cultured in the presence of $^{13}C_6$-4HB, and not with $^{13}C_6$-resveratrol (Fig. 6B), suggesting that step(s) at which resveratrol is used as a ring precursor may not depend on its conversion to 4HB, or that the production of 4HB from resveratrol is slow compared with the step where OP is utilized.

Given the structural similarity of resveratrol with p-coumarate, we tested the ability of yeast to utilize $^{13}C_6$-coumarate as a ring precursor of $^{13}C_6$-Q_6. Yeast wild-type BY4741 cells were cultured in SD-complete medium with 7, 70, or 700 μM of either $^{13}C_6$-4HB or $^{13}C_6$-coumarate for 24 h (**Fig. 7A**). We found that while the total amount of Q_6 did not change with different amounts of $^{13}C_6$-coumarate, the amount of $^{13}C_6$-Q_6 increased with higher concentrations of $^{13}C_6$-coumarate, although the incorporation was lower as compared with $^{13}C_6$-4HB. U251 human cells were labeled

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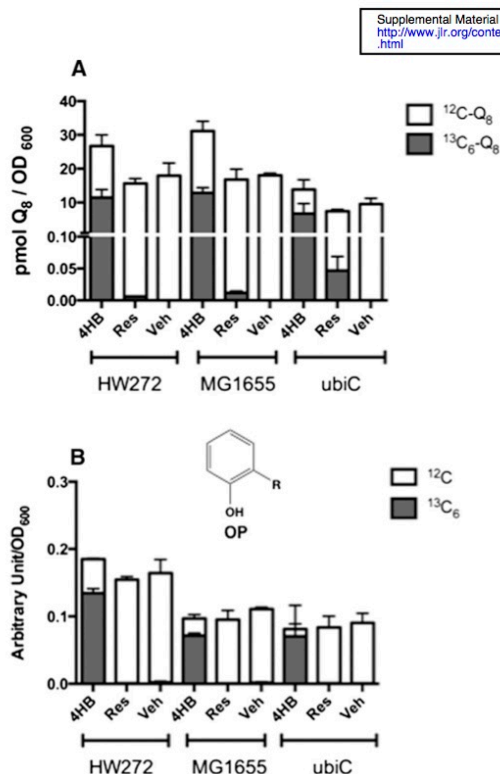

Fig. 6. *E. coli ubiC* mutant cells utilize resveratrol as a ring precursor to Q biosynthesis. Wild-type HW272, GM1655, and mutant MG1655*ubiC* cells were cultured in LB medium in the presence of 10 μg/ml of $^{13}C_6$-4HB, $^{13}C_6$-resveratrol, or ethanol as vehicle control (Veh). Collected cells were extracted and analyzed by RP-HPLC-MS/MS as described. Each bar represents mean ± SD (n = 4). The dark bar of each column represents $^{13}C_6$-Q_8 (A) or $^{13}C_6$-OP (B), while the white bar of each column represents ^{12}C-Q_8 (A) or ^{12}C-OP (B).

with 7, 70, or 700 μM of either $^{13}C_6$-4HB or $^{13}C_6$-coumarate for 24 h (Fig. 7B). We found that more $^{13}C_6$-Q_{10} accumulated when U251 cells were treated with increasing concentrations of $^{13}C_6$-coumarate. Similar to yeast cells, U251 cells showed enhanced conversion of $^{13}C_6$-4HB to Q as compared with $^{13}C_6$-coumarate. Finally, we investigated the conversion of p-coumarate to Q_8 in *E. coli*. The designated wild-type *E. coli* strains and the *ubiC* mutant were labeled with 15 μg/ml $^{13}C_6$-coumarate for 24 h. $^{13}C_6$-coumarate was converted to $^{13}C_6$-Q_8 much more efficiently in *ubiC* mutants than in the wild-type *E. coli* strains (Fig. 7C). The results show that p-coumarate is a ring precursor for Q biosynthesis in *S. cerevisiae*, *E. coli*, and human cells.

DISCUSSION

Most schemes of Q biosynthesis continue to depict 4HB as the "sole" aromatic ring precursor. The finding that *S. cerevisiae* cells could utilize pABA as a ring precursor in Q biosynthesis was rather surprising because pABA is a crucial intermediate in folate biosynthesis (9, 18). The addition of pABA to either *E. coli* or human cells leads to a concentration-dependent inhibition of Q content (4, 32, 33). Another aromatic ring compound, 4-nitrobenzoic acid, inhibited Q biosynthesis in mammalian cells by competing with 4HB for Coq2 (34). While pABA does not function as a ring precursor of Q in *Arabidopsis* (15), it remained possible that pABA might still be utilized as a ring precursor in Q biosynthesis in human and *E. coli* cells. Therefore, we employed $^{13}C_6$-pABA to investigate its fate in human and *E. coli* cells.

Treatment of cells with $^{13}C_6$-pABA revealed that pABA was not an aromatic ring precursor to Q biosynthesis in either human or *E. coli* cells. In order to rule out the scenario that *E. coli* cells might utilize pABA as a ring precursor in Q biosynthesis only when the primary ring precursor 4HB is not available, we incubated *ubiC* mutants, which have defects in the de novo synthesis of 4HB in the presence of $^{13}C_6$-pABA. However, even *ubiC* mutants were not able to utilize pABA for Q_8 biosynthesis. Interestingly, we detected multiple nitrogen-containing intermediates that derived from $^{13}C_6$-pABA. Detection of $^{13}C_6$-OAB in all three strains (HW272, MG1655, and *ubiC*) confirmed $^{13}C_6$-pABA uptake (Fig. 3A). Further modifications of the $^{13}C_6$-OAB resulted in $^{13}C_6$-OA and $^{13}C_6$-OAP, indicating UbiA, UbiD/UbiX, and UbiI tolerated the amino ring substituent (Fig. 3B, D) (21). OAP accumulated in the *ubiC* mutant independent of $^{13}C_6$-pABA addition, suggesting that OAP could be a dead-end product derived from endogenously produced unlabeled pABA. Neither HW272 nor MG1655 wild-type *E. coli* accumulated significant amounts of OAP, indicating that *E. coli* cells tend to process pABA through early steps in the Q biosynthetic pathway when 4HB content is low. These observations are consistent with studies that showed an *E. coli* mutant that lacked chorismate synthase converted pABA to OAB when cultured without addition of 4HB (33). We did not detect further downstream nitrogen-containing Q biosynthetic intermediates using targeted and limited-untargeted LC-MS/MS approaches. However, the presence of additional N-containing Q intermediates downstream of OAP cannot be ruled out.

It was shown that *Lithospermum erythrorhizon* cell cultures are able to synthesize 4HB from p-coumarate (35) and *A. thaliana* uses p-coumarate to synthesize Q (15). Therefore, we investigated whether p-coumarate is a ring precursor for Q in different organisms. We found that yeast, *E. coli*, and human cells can derive Q from p-coumarate. This finding will help us understand how 4HB is generated in these organisms. In *A. thaliana*, p-coumarate is activated by CoA ligase and the aliphatic chain is shortened to 4HB in peroxisomes (15). Because yeast, human cell cultures, and *E. coli* can use p-coumarate to make Q, it is possible that these organisms derive 4HB from p-coumarate in a similar manner.

A wide spectrum of activities is attributed to stilbenoids produced by a variety of plants when under attack by pathogens (36). A stilbenoid of recent fame, resveratrol,

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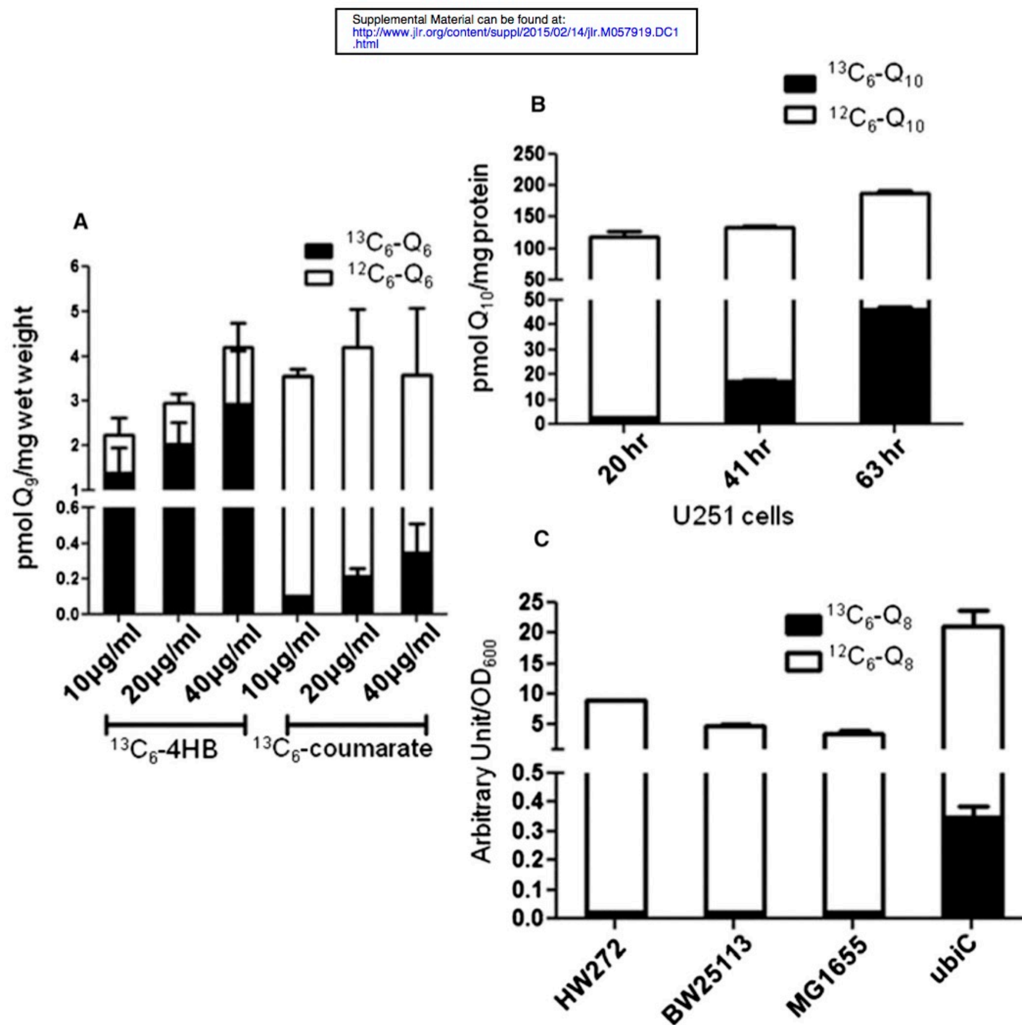

Fig. 7. $^{13}C_6$-p-coumarate is a ring precursor of Q biosynthesis in yeast *S. cerevisiae*, human cells, and in the *E. coli ubiC* mutant. A: Yeast BY4741 wild-type cells were incubated in SD-complete medium with 7, 70, or 700 μM of either $^{13}C_6$-4HB or $^{13}C_6$-coumarate for 24 h. B: U251 cells were cultured in medium with 1% FBS in the presence of 7, 70, or 700 μM $^{13}C_6$-coumarate for 24 h. C: Wild-type HW272, HW25113, GM1655, and mutant MG1655*ubiC* cells were cultured in LB medium in the presence of 15 μg/ml of $^{13}C_6$-coumarate for 24 h. Collected cells were extracted and analyzed by RP-HPLC-MS/MS as described in the Materials and Methods. The dark bar of each column represents $^{13}C_6$-Q and the white bar indicates ^{12}C-Q. Each bar represents mean ± SD (n = 4).

acts as a chain-breaking antioxidant, modulates cellular antioxidant enzymes and apoptosis, and has beneficial effects on neurodegenerative and cardiovascular diseases, eliciting metabolic responses similar to dietary restriction (37, 38). Although there is much controversy regarding the lifespan extension effects of resveratrol (39), its effects on age-associated diseases in animal models has generated considerable enthusiasm for research on its mechanism of action (40). Many questions remain regarding resveratrol biodistribution, its metabolism, and the biological effects of resveratrol metabolites (41). The beneficial health effects of resveratrol have led to vigorous research investigating its mechanisms of action.

Here we show that resveratrol serves as an aromatic ring precursor in Q biosynthesis in *E. coli*, yeast, and mammalian cells. Wild-type *E. coli* barely utilized resveratrol for Q biosynthesis; however, significant incorporation of the resveratrol ring into Q_8 was observed in *ubiC* mutants. Preferential incorporation of alternate ring precursors in the *E. coli ubiC* mutant strain is presumably due to the defect in synthesis of 4HB. In contrast, approximately 10% of the total Q content in human and mouse

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cells harbored the ring derived from $^{13}C_6$-resveratrol after 24 h of incubation. The maximum concentration of resveratrol tested was lower than either 4HB or pABA because resveratrol induced apoptotic cell death (42). Thus, we monitored cell viability in our experiments and limited the amount of resveratrol tested in order to avoid induction of cell death.

The metabolism of resveratrol responsible for its incorporation into Q has not been determined. Animals harbor two carotenoid cleaving enzymes, BCO1 and BCO2, and both are homologs of the carotenoid cleavage oxidase family (43). BCO1 is cytosolic and is responsible for cleaving β-carotene to form two molecules of retinal, while BCO2 is located in the inner mitochondrial membrane and acts on xanthophylls (44). It is tempting to speculate that BCO2, which has broader substrate specificity, might possibly cleave stilbenoids to produce two ring aldehyde products. Other family members of carotenoid cleavage enzyme in bacteria and fungi cleave resveratrol to produce 4-hydroxy-benzaldehyde and 3,5-dihydroxy-benzaldehyde (45, 46). Notably the 4′ hydroxyl group of resveratrol has been identified as crucial for antioxidant and neuroprotective effects of stilbenoids (47). It seems likely that other stilbenoids may serve as ring precursors of Q. For example, processing of piceatannol (*trans*-3,5,3′4′-tetrahydroxystilbene) by a fungal carotenoid cleavage oxidase family member (48), generates 3,4-dihydroxybenzaldehyde, a ring precursor that could potentially bypass the Coq6 hydroxylase step of Q biosynthesis upon Coq2-prenylation (49).

Of the more than one hundred clinical trials testing the efficacy of resveratrol or other polyphenols (clinical trials.gov), few determine the metabolic fate of the administered supplement. When metabolism of resveratrol is studied, the focus is on aqueous soluble polar metabolites of resveratrol, including sulfated and glucuronidated conjugation products (50). The new finding that a metabolic conversion of resveratrol into Q occurs in eukaryotes shows that exogenous antioxidants may be utilized as precursors to synthesize a wholly different class of molecule. The effects of resveratrol in mimicking calorie restriction (37, 38) may be due in part to its conversion to Q, a lipid known to induce anti-inflammatory responses (51), an essential component of mitochondrial energy metabolism, and a potent lipid soluble antioxidant (4). Investigation of the pharmacological responses to diverse dietary polyphenols (e.g., curcumin) should be expanded to include this molecular fate. Further investigation on this subject will give us a better understanding on the origin of the benzenoid moiety of Q in different organisms.

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Chapter 6

Perspectives

This work describes progress made towards characterizing the stabilizing effect of Coq8 and exogenous Q and the function of Coq9. In Chapter 2, a new model for the Q-biosynthetic complex, the CoQ-synthome, was proposed (1). Using two-dimensional blue-native/SDS PAGE to separate the digitonin extracts of mitochondria and Western blot to detect the proteins, we studied the Q-biosynthetic complex in different yeast *coq* null mutants. We found that over-expression of Coq8 (Coq8 OE) stabilizes the Q-biosynthetic complex, but the stabilizing effects are different in different null mutants. Coq8 OE restores the complex to high molecular weight in *coq3, coq5,* and *coq7* null mutants, but not in *coq4* null, suggesting Coq4 is a central organizer of the complex. The deletion of *COQ6* and *COQ9* did not cause the destabilization of the complex, so the stabilizing effect of Coq8 OE in these mutants is not as dramatic as in other mutants. Therefore, Coq6 and Coq9 are peripheral components of the complex. We also found that in *coq7* null mutant, Coq8 OE restored Coq4 to high molecular weight, but not Coq9, indicating Coq9 is associated with the complex through Coq7.

Coq8 is important for the phosphorylation of Coq3, Coq5, and Coq7 (2), and its putative kinase activity was proposed to be essential for its stabilizing effect in the *coq* null mutants (3). However, there is not yet any evidence showing yeast Coq8 is a kinase. Dr. Letian Xie had tried to purify Coq8 for *in vitro* kinase assay, but he was not able to purify soluble Coq8. It will be an important project to optimize the purification methods to obtain soluble Coq8 for kinase assay. Coq8 had not been detected in direct association with the CoQ-synthome until recently. Using the consecutive non-denaturing affinity purification, Coq8 was shown to co-purify with tagged Coq6, along with other Coq proteins, such as Coq4, Coq5, Coq7, and Coq9 (4). Coq8's association with the Q biosynthetic complex gives us a new perspective on how Coq8 stabilizes the CoQ-synthome. It will be interesting to generate a tagged functional form of Coq8 and use

non-denaturing affinity purification followed by mass spectrometry to identify associated kinase activity or any potential partner proteins that may function as kinases.

Another finding described in Chapter 2 is the effect of Q_6 supplementation on mitochondrial protein levels and Q intermediates (1). Exogenous Q_6 increases the levels of Coq4, Coq7, and Coq9 in different *coq* null mutants, but not in wild-type yeast. The addition of exogenous Q_6 also led to the accumulation of late-stage intermediates in *coq* null mutants; for example, DMQ_6 in *coq7* null, HHAB in *coq4* and *coq6* null mutants and $IDMQ_6$ in *coq9* null yeast. The results indicate that exogenous Q_6 stabilizes the CoQ-synthome. Exogenous Q_6 also increased the levels of the mitochondrial proteins Atp2, Rip1, and Mdh1, so it is possible that the addition of Q_6 improved the quantity of mitochondria in *coq* null mutants. The mitochondrial fusion protein Mitofusin 2 (MFN2) is required for mitochondrial outer membrane fusion and for maintaining mitochondrial Q levels. Mutations in the *Mfn2* gene cause defects in mitochondrial respiration and reduced ATP production, but the defects can be partially rescued by Q_{10} supplementation (5). These results suggest Q_{10} supplementation can be a treatment for diseases caused by the loss of MFN2 function. Comparing the number and morphology of mitochondria in *coq* null mutants with or without the presence of exogenous Q_6 using microscopy could be a project that helps us understand the mechanism of Q_{10} dietary supplementation treatment for patients with Q_{10} deficiency.

The function of Coq9 is not clear, but it was shown that *coq9* null yeast with Coq8 OE accumulates $^{13}C_6$-$IDMQ_6$ if $^{13}C_6$-pABA is provided as the aromatic ring precursor (3). In Chapter 3, I tested the hypothesis that Coq9 regulates the deamination of imino-containing Q intermediates (6). The yeast *coq5* point mutant *coq5-5* accumulates $DDMQ_6$ and $IDDMQ_6$, and the deletion of *COQ9* in *coq5-5* led to the disappearance of $DDMQ_6$ and the accumulation of

imino-containing Q intermediates, $IDDMQ_6$ and 4-AP. A temperature-sensitive *coq9* point mutant, *coq9-ts19*, was also generated to study the function of Coq9. This mutant has defective growth on respiratory media at non-permissive temperature. The expression of *coq9-ts19* decreased the steady state levels of other yeast Coq polypeptides, such as Coq4, Coq5, Coq6, and Coq7, indicating the destabilization of the Q biosynthetic complex. The changes in Coq polypeptides were suggested to occur at the protein level because there is no corresponding change in *COQ* RNA levels. We also found that the *coq9* point mutant has a decreased level of Q_6 and increased levels of imino-containing Q intermediates, $IDDMQ_6$ and 4-AP. We concluded that yeast Coq9 controls the removal of the nitrogen group of Q intermediates derived from pABA.

Whether Coq9 is a deaminase requires further investigation. Bypass experiments with 4-hydroxybenzoic acid analogues had been conducted. It was shown that 2,4-dihydroxybenzoic acid significantly increased the Q levels in a mouse model *Coq9*^{R239X} that recapitulates the human R244X mutation (7). If yeast Coq9 is a deaminase, then 2,4-dihydroxybenzoic acid will be able to bypass *coq9* mutations, but not 4-aminosalicylic acid or 4-amino-2-methoxybenzoic acid. Dr. Letian Xie and Alice Hsu fed these compounds to *coq7* and *coq9* yeast mutants with or without Coq8 OE and analyzed the lipid extracts with LC-MS/MS. The positive control *coq7* null with Coq8 OE was able to produce Q_6 with all of these precursors. However, *coq9* null with Coq8 OE only produce a very small amount of Q_6 with 2,4-dihydroxybenzoic acid. Coq9 is important for Coq6's function, so Alice Hsu and I tested whether 2,3,4-trihydroxybenzoic acid can rescue *coq9* null with Coq8 OE, and the result was negative. It is possible that Coq9 is required for other steps in the Q biosynthesis pathway. In this case, we can try to use these compounds to bypass the yeast point mutant, *coq9-ts19*. The temperature-sensitive mutant still produces Q_6 at non-

permissive temperature, so there is functional Q biosynthetic complex. After feeding the above compounds to *coq9-ts19* and grow the yeast cultures at permissive or non-permissive temperatures, we can compare *coq9-ts19*'s responses to the bypass treatments with different compounds. If our hypothesis is correct, we will see a significant increase of Q_6 production at non-permissive temperature when the mutant yeast was fed with 2,4-dihydroxybenzoic acid or 2,3,4-trihydroxybenzoic acid, but not with 4-aminosalicylic acid or 4-amino-2-methoxybenzoic acid. Alice Hsu fed 2,4-dihydroxybenzoic acid to BY4741Δ*coq9* expressing *coq9-ts19* with or without Coq8 OE, but she did not see an increase of Q_6 level in response to the bypass treatment. The reason she used a *coq9* null in BY4741 background is because it allowed her to express both the TS19 and the multi-copy *COQ8* plasmids. However, I have encountered some problems with this strain. I found that the expression of wild-type Coq9 rescued W303Δ*coq9* at non-permissive temperature but not BY4741Δ*coq9*. Therefore, the negative results we saw with BY4741Δ*coq9* might be strain specific. I have generated a *coq9* null in W303 background (W303Δ*coq9*K) that is both Ura^- and Leu^- so it can express both TS19 and the multi-copy *COQ8* plasmids. The bypass experiments can be repeated with this new strain.

Although $IDDMQ_6$ and 4-AP accumulate in *coq9* mutants, whether they are productive intermediates remains a question. Synthesized imino-demethoxy-Q_3 or 3-triprenyl-4-aminophenol can be added to yeast cultures and determine whether Q_3 is synthesized by analyzing lipid extraction with LC-MS/MS. If the results are negative, it is possible that the uptake efficiency by yeast cells is low. In this case, these compounds can be added to purified mitochondria instead of yeast cultures. The reduced form of imino-demethoxy-Q_3 can also be tested and purified mitochondria will be a better choice in case oxidation takes place before cells uptake the compound.

Coq9 is required for Q_{10} biosynthesis in human and its function is still unknown. Based on a protein sequence alignment I performed on NCBI blast, there is only 26% identity shared by yeast Coq9 and human Coq9. However, yeast and human Coq9 share some functions. *Coq9* mutant mouse that recapitulates the R244X human *coq9* mutation has decreased level of COQ7 polypeptide and accumulation of demethoxy-Q_9 (8). In yeast, Coq9 is important for the function of Coq7 and demethoxy-Q_6 accumulates in yeast *Coq9* mutant. Therefore, we investigated whether human COQ9 complements yeast *coq9* mutants. In Chapter 4, I showed that human COQ9 rescues the temperature-sensitive *coq9* point mutant, *coq9-ts19*. In contrast, human COQ9 failed to rescue yeast *coq9* null mutant even with Coq8 OE. It is possible that yeast Coq9 is required for the function of Coq6 and Coq7, so we expressed human COQ9 in *coq9-ts19*, a mutant that has decreased but functional yeast Coq proteins. We found that human COQ9 increased the growth of *coq9-ts19* on respiratory media, stabilized different yeast Coq polypeptides, and elevated Q_6 content by enhancing Q_6 biosynthesis from 4-HB. The fact that human COQ9 is better in incorporating 4-HB into Q_6 than utilizing pABA when it is expressed in *coq9-ts19* suggests that human COQ9 does not have the function of mediating deamination like yeast Coq9 does. Interestingly, pABA is a precursor for Q in yeast, but not in human cells. It is likely that yeast Coq9 is the key factor for yeast's ability to produce Q_6 from pABA. To investigate the mechanism for the rescue by human COQ9, we expressed human COQ9 in a yeast strain containing tagged Coq6 and performed immunoprecipitation. We found that human COQ9 co-purified with yeast Coq6, indicating the association between human COQ9 and the CoQ-synthome. For the first time, there is a successful rescue of yeast *coq9* mutant with human COQ9. It not only gives us insights to the differences between yeast and human Q biosynthesis, but also enables us to use yeast as a model for future studies on the function of human COQ9.

Although I have shown that human Coq9 is associated with yeast Coq6, it will give us more information on how human Coq9 rescued the yeast *coq9* mutant if we investigate what lipids or other proteins interact with human Coq9. There are commercially available kits that can help us to prepare columns for affinity purification without going through cloning to generated tagged human Coq9. For example, the AminoLink Plus Coupling Resin (Thermo Scientific) is aldehyde-activated beaded agarose that functions to conjugate antibodies. Therefore, we can use this product to covalently attach human Coq9 antibodies to the resin and then perform immunoprecipitation with mitochondria purified from yeast *coq9* mutant expressing human COQ9. The eluate can be analyzed with mass spec to identify lipids and proteins that are associated with human Coq9 or with SDS-PAGE followed by Western blot to determine whether other yeast Coq polypeptides co-purify.

In Chapter 5, I described the investigation on the role of coumarate as an aromatic ring precursor in Q biosynthesis. I found that when $^{13}C_6$-coumarate was fed to *E. coli*, *S. cerevisiae*, and human cell cultures, $^{13}C_6$-Q was detected in lipid extracts by LC-MS/MS. The *E. coli* strain *ubiC* mutant produced $^{13}C_6$-Q_8 from $^{13}C_6$-coumarate more efficiently than the wild-type *E.coli* strain. It is likely that the mutant uses more $^{13}C_6$-coumarate as a ring precursor because it cannot synthesize 4HB, usually a preferred Q precursor. We conclude that coumarate serves as a ring precursor for Q biosynthesis in *E. coli*, *S. cerevisiae*, and human cells.

In *Arabidopsis thaliana*, phenylalanine was first converted to coumarate, which is transported from the cytosol to peroxisome. Coumarate is then ligated to CoA producing p-coumaryl-CoA, which was then used to produce 4HB (9). It was proposed that coumarate is converted to 4HB in yeast in a similar manner (10), but enzymes involved in this pathway are unknown. We had hypothesized Pcs60, a peroxisomal CoA-dependent synthetase, as the enzyme

that converts coumarate to coumaryl-CoA in yeast. To test this hypothesis, I compare the incorporation of $^{13}C_6$-coumarate and $^{13}C_6$-4HB in wild-type yeast and *pcs60* null mutant yeast. In several experiments, the *pcs60* null mutant appeared to be defective in synthesizing $^{13}C_6$-Q_6 from $^{13}C_6$-coumarate, but the results were not consistent in subsequent experiments. An explanation is that *PCS60* is not essential in Q biosynthesis from coumarate. However, the $^{13}C_6$-coumarate used in those experiments was not purified. It is possible that a small amount of contamination containing $^{13}C_6$-aromatic rings also contributed to the amount of $^{13}C_6$-Q_6 we measured. The $^{13}C_6$-coumarate used in Chapter 5 had been purified, so it will be important to repeat the experiments with the pure precursor.

The work in this dissertation describes the projects elucidating the steps of Q_6 biosynthesis pathway and the functions of proteins involved. The study on the effects of over-expression of *COQ8* and supplementation of Q_6 gives us a better understanding on the CoQ-synthome and how exogenous Q_6 rescues yeast *coq9* null mutants. The function of Coq9 has been a mystery and in this study we had demonstrated that Coq9 regulates the deamination steps in Q_6 biosynthesis pathway. For the first time, we had successfully rescued *coq9* yeast mutant with human *COQ9*, making yeast a potential model to study the function of human Coq9. Lastly, the findings on *p*-coumarate serving as a Q ring precursor give us a better understanding on the origin of the benzoquinone ring of Q.

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